

THE RATE AT WHICH SPERMATOGENESIS OCCURS IN THE RABBIT

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ONE PLATE (SIX FIGURES)

INTRODUCTION

An important unsolved problem of testicular physiology is the rate of spermatogenesis. Under normal conditions a continuous supply of spermatozoa is available for passage to the epididymis and since the length of the seminiferous tubules is very great, the assembly line produces an adequate supply. Phillips and Andrews ('36) have obtained a rough approximation for the ram, bull and boar. They castrated males at various ages, one at each age studied. In rams at 84 to 105 days of age primary spermatocytes were present, at 126 days secondary spermatocytes, and at 147 days spermatozoa were formed. In bulls at 104 days primary spermatocytes were present, secondary spermatocytes at 181 days and spermatozoa at 224 and 261 days. In the boar primary spermatocytes were present at 84 days, secondary spermatocytes at 105 days and spermatozoa at 147 days. These rates of development are slower than those found by us for the rabbit, but they refer to growing males and not to mature ones. The rate of development of spermatozoa from spermatocytes in the ram, 21 days, closely resembles our result, about 3 weeks or more, for the similar stages in the rabbit.

Our method has been to anchor the testes of year-old bucks in the abdomen for a definite time, then to remove one testis as a control and to return the other to the scrotum. After a

suitable interval it was removed and sectioned. We have been able to gather data by this method, not only on the rate of spermatogenesis in the rabbit, but also on the rate of degeneration of the testis in the body cavity and the interval required in the scrotum before regeneration sets in.

A good deal of work has been done upon the physiology and histology of the testis placed in the abdomen. There is fairly general agreement that the observed effects are due to the higher temperature in the body cavity. Moore ('24-'25) has described the effects produced by the retention of the testes of the guinea pig in the scrotum for 7 days. Knaus ('32) has described similar effects in the rabbit and our results are in agreement with these workers. Apparently no one has studied the effects of a stay of less than 7 days in the abdomen. Moore ('24-'25) records the fact that regeneration occurs if the testes are replaced in the scrotum.

EXPERIMENTAL

As so much is known of the behavior of the guinea pig abdominal testis, this species was first chosen for the work. However, it was found that, due probably to the very shallow scrotum and the wide inguinal canal, it was difficult to keep the returned testis in the scrotum, so that after fifteen trials in which the number of successes was but seven, the rabbit was tried. It was found that the testes merely pushed through the inguinal canal into the abdomen usually stayed up, but the degree of success was not great enough to make this a reliable method. Accordingly the bucks were laparotomized under nembutal or ether anesthesia and the testes brought up into the abdomen and anchored to the rectus muscle by a thread of silk, passing loosely through the fat body at the head of the epididymis. Care was taken not to include blood vessels or epididymal structures within the loop. After the required number of days another laparotomy was performed and the testes were released. One was excised to determine the degree of degeneration which had occurred, while the other was returned to the scrotum by traction on the scrotum and by

pushing down the testes from within as gently as possible. At first attempts were made to anchor the testes in the scrotum by a ligature through the scrotal wall or across the inguinal canal, but as this caused the onset of hydrocele, the method was abandoned and the testes left in the scrotum without any anchorage. They stayed down very well except in the bucks in which they had remained in the abdomen for 4 or 5 days or more. Apparently the spermatic cord had shortened by this time so that it was difficult for the testes to remain down. Accordingly many more experiments were done at these times to fill in the gaps. In two rabbits the spermatic cords were tied off so that we should know the appearance of a necrotic testis. The testes of a rabbit vasectomized for 18 months were also sectioned to familiarize us with their appearance.

The testes were kept in the abdomen for 1, 2, 3, 4 and 5 days, and a few for a month. The subsequent time in the scrotum was for much longer periods. Recovery was very slow for testes kept up for more than 5 days.

Group 1. Testis retained in body cavity for 1 day

In these rabbits, ten in all, it was found that the main immediate result of keeping the testis for 1 day in the abdomen was to cause the spermatids to become clumped and detached. In three cases the spermatocytes showed some derangement and lacunae. It was evident in all cases that spermatogenesis had ceased and that no further mitoses were in progress even in the spermatogonial layer.

In the testes returned to the scrotum for 7 days the degeneration had gone further, the spermatozoa and spermatids had disappeared, the spermatocytes were clumped and the layer of spermatogonia showed some signs of damage. It is evident that the damage done by a 1 day stay in the body cavity is not at once obvious but that several days are necessary for it to be fully apparent.

Group 2. Testes retained in the body cavity for 2 days

After a stay of 2 days in the abdomen, the testes had a somewhat variable appearance. In twelve rabbits the sperm

had disappeared or (in two cases where they were still present) showed signs of disintegration. The spermatids were either clumped or had sloughed off. In three cases the spermatocytes had joined in the clumping. In two testes there were rare signs of mitoses, the only rabbits in the experiment to show this state, which appears to be much rarer than it is in the guinea pig at 7 days (Moore, '24-'25). In one case giant cell formation was in progress.

Group 3. Testes retained in the body cavity for 3 days

In eleven specimens observed after the testes had been in the abdomen for 3 days, the results were fairly uniform. In five, sperm heads were found, but in all the spermatid layer was unrecognizable; the spermatocytes were clumped with lacunae in this layer. Giant cells were observed in most testes.

Group 4. Testes retained in the body cavity for 4 days

The general results for ten specimens after a stay of 4 days in the abdomen were the same as for 3 days; in fewer specimens were spermheads recognizable, while the spermatocyte layer seemed to be a little more degenerated.

Group 5. Testes retained in the body cavity for 5 days

After a stay of 5 days in the body cavity (ten specimens) the degree of degeneration was almost extreme. It corresponded very closely with Moore's ('24-'25) account of the degeneration produced in testes retained in the body cavity for 7 days.

The rates of recovery of the testes in fifty-two bucks after they were returned to the scrotum are given in table 1. The time at which recovery set in varied roughly with the duration of their stay in the abdomen, except for those which were kept in the abdomen for 4 days. These showed an unaccountable lag. There was fair agreement in the time taken for the establishment of the various layers in the seminiferous tubules, about a week for each layer and 2 weeks for the development

of tails after the spermatids had migrated to the cells of Sertoli. Three further experiments in which the testes were retained in the abdomen for a month or more showed no signs of returning activity after 2 months in the scrotum, so that the return of activity after a prolonged exposure to the unfavorable environment is very slow.

TABLE 1
Testis regeneration after stay in abdomen

TESTES IN ABDOMEN	TESTES SUBSEQUENTLY IN SCROTUM, DAYS						
	7	14	21	28	35	42	49
1 day	O O	C	I+	T T	O S S+ S++		
2 days	O O	C	C	I+ I	I O C O+ O	I	
3 days	O O+	O+	O+ O	C O+ O+	I+ I+	O	
4 days	O		C	O+ O+	O+ O	O O	O
5 days	O	O+ C	O+ O+	C+	O+ I	T	O

O = single layer, no activity.

O+ = mitoses in spermatogonia.

C = spermatocytes.

C+ = very active spermatocyte layer.

I = spermatids.

T = tail formation.

S = spermatozoa formed.

S+ and S++ = numbers of spermatozoa.

DISCUSSION

The course of testis degeneration is the same for the rabbit as for the guinea pig. Even a stay in the body cavity for 1 day does very considerable damage, and the damage is not fully apparent at the end of 1 day but continues after the testis is

returned to its normal environment. The ultimate damage does not appear to be quite so great as it is with a longer stay, for 2 days up to 5 days in the body, the limit in the detailed part of this work. The rate of recovery is roughly proportional to the length of the stay in the body cavity. The longer the testes remain up, the slower their recovery.

Roughly, the establishment of active cells in each layer requires about 1 week, and the growth of tails to the spermatids requires about 2 weeks. We cannot say, of course, that this is the rate in normal life, where spermatogenesis is a continuous process, travelling in waves along the seminiferous tubules, but it does tend to set an upper limit for the rate of spermatogenesis, a conclusion that has hitherto been lacking. We hope that it will be possible eventually to work out a better technique than the somewhat rough qualitative method which we have employed.

The objection may be raised that these results are affected by compensatory hypertrophy of the one remaining testis. But recent work has shown that compensatory hypertrophy of the testis does not occur (Edwards, '40). The testis differs from the ovary in this respect.

SUMMARY

1. Testes which had been placed in the abdomen for various times were returned to the scrotum. The degree of degeneration and the rate of regeneration were observed.

2. A stay of 1 day in the abdomen produces considerable damage which is not fully apparent at the end of the 24 hours, as it continues after the testes are returned to the scrotum. A stay in the abdomen longer than 1 day produces somewhat more damage.

3. The rate of recovery of the testis after its return to the scrotum is roughly proportional to the length of its stay in the abdomen.

4. Approximately 1 week is needed for the establishment of each successive layer in the seminiferous tubules, and 2 weeks for the spermatids to grow their tails.

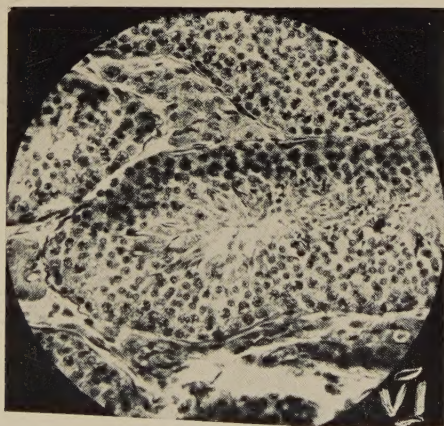
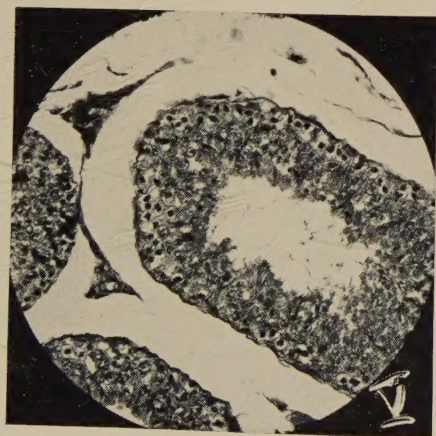
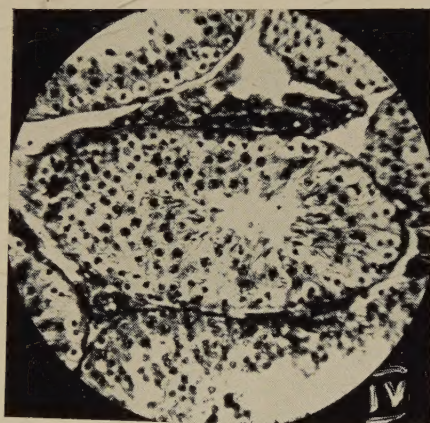
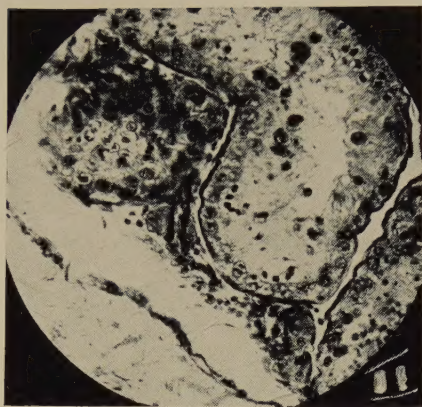
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PLATE 1

EXPLANATION OF FIGURES

- 1 Testis in abdomen 1 day. The spermatids are clumped and detached. $\times 250$.
- 2 Testis in abdomen 1 day, then returned to scrotum for 7 days. The further degeneration has included the spermatogonia layer. $\times 250$.
- 3 Testis in abdomen 1 day, then returned to scrotum for 21 days. Regeneration has proceeded to the beginning of the spermatid stage. $\times 250$.
- 4 Testis in abdomen 2 days, then returned to scrotum for 30 days. Active spermatid formation. $\times 250$.
- 5 Testis in abdomen 1 day, then returned to scrotum for 39 days. Commencement of tail formation on spermatids. $\times 250$.
- 6 Testis in abdomen 1 day, then returned to scrotum for 39 days. Well developed spermatozoa. $\times 250$.



FACTORS UNDERLYING THE FAILURE OF CYCLIC MATING BEHAVIOR IN THE ALBINO RAT ¹

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ONE PLATE (FOUR FIGURES)

The experience of the writers has been that when any randomly selected group of guinea pigs or albino rats is placed under observation for the display of cyclic mating behavior a small percentage of animals is found in which heat is of short duration or of irregular occurrence if it occurs at all. In addition, the failure of pregnancy in females confined continuously with normal males must frequently be accounted for by the failure of the female to become sexually receptive. In guinea pigs of this type which came under observation the failure of heat seemed attributable to poor follicular development, or to the failure of luteinization and ovulation at the expected time, or, in animals in which the ovarian condition was normal, to a high threshold to the conditioning action of estrogen (Young, Dempsey, Myers and Hagquist, '38; Boling, Young and Dempsey, '38).

These studies involved techniques which were widely separated in point of time and when the results were summarized and it was realized that factors in reproductive failure had been identified, the animals were no longer available for observation and experiment. It would have been desirable to know if the types of hypophyseal failure which were encountered

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were transitory or likely to be permanent, if any connection exists between such hypophyseal deficiencies and the sensitivity to estrogen, and if larger quantities of estrogen than those used would condition the most unresponsive animals for heat. Of equal interest was the extent to which the same factors might account for the failure to mate in other species.

An opportunity to investigate these problems was presented when the usual residuum of abnormal rats had accumulated at the end of the observations described elsewhere (Blandau, Boling and Young, '41; Boling, Blandau, Soderwall and Young, '41; Young, Boling and Blandau, '41) and after the normal rats from a second colony had been selected for artificial insemination in an independent experiment by Blandau and Jordan ('41).

MATERIALS AND METHODS

Out of 457 adult female rats which had been under observation from 5 weeks to 6 months, thirty-two were found in which heat was occurring only irregularly or in which there was no evidence that it had ever occurred. Twenty-one which did not subsequently display heat were used in this study. Thirteen were from 8 to 12 months old when the study was begun, October 19, 1939. Of the thirteen, eight had been observed for signs of heat at hourly intervals, day and night, during the period, May 14 to June 18, 1939, and five had been confined with normal males during the period, December 20, 1938 to June 15, 1939. The observations which were made during the period, October 19 to November 7, 1939, extended throughout an equivalent of four reproductive cycles and provided confirmatory evidence that heat was not being displayed or, at the best, was occurring only irregularly. Twice daily during the period, October 30 to November 7, vaginal smears were made.

Each animal was unilaterally ovariectomized November 7. The ovaries were fixed in Zenker's fluid without acetic acid, sectioned and stained with Delafield's hematoxylin and eosin. From December 16 to 21, inclusive, the animals were again observed for heat, and vaginal smears were made twice daily.

Immediately thereafter the second ovary was removed and prepared for microscopic study. The spayed animals were then ready for tests of their sensitivity to the action of estrogen and progesterone, and nine times, at intervals of not less than 8 days, they were injected subcutaneously with varied quantities of estradiol benzoate (Progynon-B) followed by progesterone (Proluton) and observed for heat.

The quantity of estrogen was increased in the third, fourth, fifth and ninth injections (table 2) in order that the results could be compared with those obtained following the first and second injections when quantities ordinarily sufficient to induce heat in a high percentage of rats were given (Boling and Blandau, '39). The smaller quantity of estrogen was used for the sixth injection in an effort to ascertain if the sensitivity had been affected by the larger quantities given in the third, fourth and fifth injections. At the time of the seventh and eighth injections the quantity of progesterone was increased in order to determine if the failure to display heat noted previously could be attributed to a higher than normal threshold for this hormone. Beginning 40 hours after the injection of estrogen and continuing until the fifteenth hour after the injection of progesterone the animals were observed for heat. The ninth series of injections was composed of four injections of 50 R.U. each of estrogen over a 48-hour period. In four animals in which these injections had not induced heat by the ninety-sixth hour, 0.4 I.U. progesterone was injected and the observations were continued. The controls consisted of a group of animals of approximately the same age which were injected with the smallest quantity of estrogen given any of the experimental animals.

The abnormality of the eight animals not yet accounted for was established by means of examinations at frequent intervals from October 10 to December 10, 1939 and by their failure to become pregnant during the period December 10, 1939 to March 29, 1940 when they were confined with normal males. Ovariectomy was performed April 17, 1940 when they were approximately 10 months old. Following a recovery period of

13 days they were injected with 10 R.U. estradiol benzoate and 72 hours later with 0.4 I.U. progesterone. A second series of injections consisted of 20 R.U. estradiol benzoate May 11, 1940 followed by 0.4 I.U. progesterone 72 hours later. Because of the shorter period of experimentation, this group of animals was less valuable than the larger group followed longer. Nevertheless, miscellaneous supporting and confirmatory data were obtained.

Grateful acknowledgment for the supply of Progynon-B and Proluton is made to Dr. Erwin Schwenk of the Schering Corporation. For indispensable assistance in observing the animals we are indebted to Messrs. William R. Fish, Edward S. Jordan, Arnold Soderwall and James G. Wilson.

EXPERIMENTAL

The thirteen animals from which the most extensive data were obtained form a heterogeneous group. The records with respect to the occurrence of heat prior to complete ovariectomy varied (table 1) and the records with respect to the occurrence of induced heat during the postoperative period of experimentation likewise varied (table 2). Conspicuous differences were found in the ovarian condition and in the vaginal smear picture. It seemed at first therefore that a tedious review of case histories could hardly be avoided. Subsequently, however, it became clear that the ovarian condition could be employed as a basis for consideration of the data.

In five animals (7, 13, 52, 150A, 164) the ovaries appear to be normal inasmuch as they contain growing follicles and several generations of normal appearing corpora lutea (fig. 1). The ovaries from one animal (152) contain numerous growing and large atretic follicles, but no corpora lutea or evidence that luteinization had ever occurred (fig. 2). They are similar to those described by Evans and Long ('21) as a "rare anomaly" and to those found in rats maintained on a diet deficient in vitamin A (Evans and Bishop, '22). More recently ovaries of this type have been described as occurring spontaneously

in a strain of rats maintained at Duke (Everett, '39). Observations by Asdell and Crowell ('35), Bryan, Klinck and Wolfe ('38) and Wolfe, Bryan and Wright ('38) indicate that the abnormality is sometimes associated with age. Comparison of these ovaries with those stimulated by purified fractions of follicle-stimulating hormone (Fevold, Hisaw and Leonard, '31; Wallen-Lawrence, '34; Rinderknecht and Williams, '39) suggests that the basic endocrine abnormality is a failure of luteinizing hormone action.

In the other seven animals (20, 33, 100, 111, 113, 134, 176) the presence in one or both ovaries of atretic, non-luteinized follicles or a generation of follicular cysts or the absence of a generation of corpora lutea suggests that occasionally there was a failure of luteinization. In addition, the poor general follicular development and the frequent occurrence of small atretic follicles in the first ovaries removed from animals 33, 100 and 176 and in both ovaries from animal 111, indicated a more general deficiency of gonadotropic action (fig. 3). Such a condition is also described by Wolfe, Bryan and Wright ('38) in the Albany strain of rats.

When the records summarized in tables 1 and 2 are examined it is clear that irrespective of the ovarian condition all the animals showed a low sensitivity to estrogen. None displayed signs of heat when 10 R.U. estrogen were followed 48 hours later by 0.4 I.U. progesterone although in the control group heat was induced in the five animals given this treatment. Its length averaged 11 hours, range 10 to 12 hours. When the experimental animals were given 10 R.U. estrogen followed 72 hours later by 0.4 I.U. progesterone, two of eleven displayed heat. In the control group the five injected animals came into heat. The length averaged 13.8 hours, range 9 to 17 hours. When the quantity of estrogen was increased to 20 R.U. followed by progesterone, three more experimental animals responded. Thereafter the number displaying heat remained about the same, even when 10 R.U. were given, until the ninth injection when all responded to 200 R.U. It is considered significant that of the animals which came into heat

following this treatment, four (100, 150A, 152, 176) are not known to have come into heat prior to ovariectomy or in consequence of the injection of smaller quantities of estrogen followed by progesterone during the experimental period. The increased dosage of progesterone in the seventh and eighth series of injections was no more effective than the smaller quantity used in the other injections.

TABLE 1

Length of heat in hours and the vaginal condition during the pre-experimental period of rats in which mating behavior was of irregular occurrence or absent

CYCLE	PREOPERATIVE OBSERVATIONS				VAGINAL CONDI- TION PRIOR TO FIRST OPERATION	INTEROPERATIVE OBSERVATIONS 1 CYCLE	VAGINAL CONDI- TION PRIOR TO SECOND OPERATION
	1st	2nd	3rd	4th			
Animal	Animals in which the ovarian condition was normal						
7	12-hr. estrus	0	0	0	Normal cyclic changes	0	Normal cyclic changes
13	0	0	2-hr. estrus	0	" "	9-hr. estrus	" "
52	0	6 hr. estrus	5-hr. estrus	13-hr. estrus	" "	0	" "
150A	0	0	0	0	Acyclic variations	0	" "
164	2-hr. estrus	0	0	0	" "	0	" "
Animals in which a deficiency of hypophyseal stimulation is indicated							
20	0	0	0	0	Normal cyclic changes	Intermittent estrus 1st part—13 hrs. 2nd part—11 hrs.	Constant cornification
33	0	0	0	0	Constant cornification	9½-hr. estrus	" "
100	0	0	0	0	" "	0	Normal cyclic changes
111	0	6 hr. estrus	0	0	" "	Ovaries removed 11/6/39	Ovaries removed 11/6/39
113	0	0	13-hr. estrus	0	Acyclic variations	0	Constant cornification
134	12-hr. estrus	7-hr. estrus	0	11-hr. estrus	" "	0	Acyclic variations
152	0	0	0	0	Constant cornification	0	Constant cornification
176	0	0	0	0	Prolonged cornification	0	Constant diestrous condition

The response to the ninth series of injections when 200 R.U. estrogen were administered is of interest from the standpoint of our experience with estrogen and progesterone. Boling and Blandau ('39) showed that as the quantity of injected estrogen

TABLE 2

Length of induced heat in hours of rats listed in table 1 during the period of postoperative estrogen-progesterone injections.

The dash is shown where no injection was made

INJECTION	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
QUANTITY OF ESTROGEN	10 R.U.	10 R.U.	20 R.U.	20 R.U.	40 R.U.	10 R.U.	40 R.U.	10 R.U.	200 R.U.
INTERVAL BETWEEN INJECTIONS OF ESTROGEN AND PROGESTERONE	72 hrs.	48 hrs.	72 hrs.	72 hrs.	72 hrs.	72 ^a hrs.	72 hrs.	72 hrs.	96 hrs.
QUANTITY OF PROGESTERONE	0.4 I.U.	0.4 I.U.	0.4 I.U.	0.4 I.U.	0.4 I.U.	0.4 I.U.	1.0 I.U.	1.0 I.U.	0.4 I.U. into animals marked *
Animal Animals in which the ovarian condition was normal									
7	8	0	12	12	8	5	12	3	> 60-hour estrogen estrus
13	8	0	7	11	0	10	—	2	* 15-hour estrogen-progesterone estrus
52	0	0	7	5	0	8	7	0	> 74-hour estrogen estrus
150A	0	0	0	0	0	0	0	0	Intermittent estrogen estrus
164	0	0	0	0	0	0	6	3	> 21-hour estrogen estrus
Animals in which a deficiency of hypophyseal stimulation is indicated									
20	0	0	9	12	8	11	—	3	Intermittent estrogen estrus
33	0	0	8	7	15	0	11	0	—
100	0	0	0	0	0	0	0	0	Intermittent estrogen estrus
111	0	0	0	0	0	0	0	—	—
113	0	0	0	0	0	0	11	0	> 77-hour estrogen estrus
134	0	0	0	0	10	0	0	0	* 5-hour estrogen-progesterone estrus
152	0	0	0	0	0	0	0	0	* 16-hour estrogen-progesterone estrus
176	0	0	0	0	0	0	0	0	* 8-hour estrogen-progesterone estrus

is increased the percentage of animals displaying an estrogen induced heat likewise increases, but not even 100 R.U. were sufficient to induce heat in all animals. Heat was induced in the resistant animals, however, by the subsequent injection of progesterone. In this experiment the result has been the same. The four animals which did not display signs of heat following the administration of 200 R.U. estrogen responded when progesterone was given. Consequently, of the animals we have handled which have not responded to estrogen, none has failed to respond to the secondarily injected progesterone. This is regarded as further evidence for the rôle of progesterone in the induction of heat in this species.

The ovarian condition has been discussed only to the extent of explaining the order in which the animals are listed in tables 1 and 2. Another aspect of the subject which is of interest is the nature of differences between the first ovary and the second ovary removed 44 days or approximately ten reproductive cycles later. When both ovaries are removed at the same time their condition, insofar as it reflects the character of hypophyseal stimulation, tends to be similar. Consequently, one is fairly safe in assuming that if the ovaries are removed at different times and their character varies, a change in the quality of the hypophyseal stimulation is indicated.

In five animals in which the first ovary was normal the second ovary was also normal. The second ovary was similar to the first in two of the seven animals in which hypophyseal stimulation appeared to be deficient, but in the other five animals of this type differences are seen which are believed to indicate that the character of hypophyseal stimulation changed during the interval between operations. Animals 152 and 134 are the two in which no change occurred. In neither ovary removed from 152 were there corpora lutea or any evidence of luteinization. In both ovaries from animal 134 corpora lutea of several generations can be seen, but the presence of a generation of follicular cysts in one ovary and a generation of atretic follicles in the other is taken as evidence that prior to the first operation as well as the second luteinizing hormone action was irregular.

Varying hypophyseal gonadal relationships are indicated for the other animals. The first ovaries removed from animals 20 and 113 were normal except for abnormalities caused by distention of the periovarial sac in animal 20. The second ovaries, on the other hand, lacked corpora lutea, thus making it appear that luteinizing action had ceased during the interval between operations. To a lesser degree the same change had occurred in animal 33. The presence of a few small, old corpora lutea in the first ovary suggests that some luteinizing hormone action had occurred prior to the removal of this ovary, but the absence of corpora lutea from the second ovary suggests that by the time it was removed luteinization had stopped entirely. General follicular development, on the other hand, was much better than in the other ovary. This may be an expression of the compensatory hypertrophy which was noted during these experiments.

The change in the two remaining animals seems to have been in the direction of normality because the appearance of the second ovary suggests that the gonadotropic action was greater when it was removed than it had been 44 days earlier. In the first ovary from animal 176 general follicular development is poor and no evidence of luteinization is seen, but by the time the second ovary was removed general follicular development had improved and a generation of normal appearing corpora lutea is present, indicating that at that time luteinization was occurring at least occasionally. In the first ovary from animal 100 general follicular development is poor and a few old corpora lutea are present, but the second ovary appears normal in every respect (figs. 3 and 4).

The conclusion to this study of the ovarian condition is that there are adult rats in which the hypophyseal gonadal relationship is not stable. The defects range from a grossly incomplete follicular development without luteinization to a follicular development which appears normal except that when the preovulatory swelling should begin, ovulation and luteinization do not occur. Although this difficultly obtained material is not sufficient to permit much by way of generalization, it

appears from what has been available for this investigation as well as that described by Everett ('39) that the degree of abnormality may vary from time to time either in the direction of normality or of greater abnormality. It appears further from this study that fluctuations in the extent of general follicular development and luteinizing hormone action vary independently. In the three animals in which the extent of general follicular development changed (33, 100, 176) an improvement is seen. On the other hand, when corpus luteum formation is used as an index of luteinizing hormone action, three animals (20, 33, 113) changed in the direction of abnormality and two (100, 176) in the direction of normality. The extent to which these abnormal conditions may be permanent is not known except that in two animals no change occurred over a period of approximately ten reproductive cycles.

The vaginal condition during the periods immediately prior to the operations is summarized in table 1. It was anticipated that normal cyclic changes would be shown by animals in which the ovarian condition was normal and constant cornification by the animals in which luteinizer action was deficient (Evans and Long, '21; Evans and Bishop, '22; Witschi and Pfeiffer, '35). In general, this expectation was realized. The most conspicuous exceptions were the vaginal conditions seen in animals 113, 150A and 164 prior to the first operation, that in animal 176 prior to the second operation and that in both sets of smears from animal 134. Cornified cells were present much of the time, but there were frequent infiltrations of leucocytes. "Pavement" cells (Young, Boling and Blandau, '41) are also seen in many preparations. The order in which the different cell-types appeared and the length of time they were present varied. When allowance is made for uncertainties with respect to the secretory activity of these ovaries it is not surprising that irregularities occurred.

The data obtained from the eight animals from which the ovaries were removed during a single operation and which were given only two series of injections are summarized in table 3. As in the larger group studied over a longer period,

the ovarian condition was normal in some animals and showed evidence of a deficiency of hypophyseal stimulation in others. The appearance of the ovaries from the two animals in which this latter condition was found was identical with that seen in the ovaries from animal 152 (fig. 2). They were devoid of corpora lutea although follicular development up to the stage at which the preovulatory swelling ordinarily begins seems to have been normal. The response to the injected hormones was irregular, but inasmuch as heat behavior was induced in animals which are not known to have been in heat previously, it is felt that for these animals as well as those in the larger group, a low sensitivity to estrogens is indicated.

TABLE 3

Ovarian condition of supplementary group of abnormal rats during the pre-experimental period, and length of induced heat in hours during the period of postoperative estrogen-progesterone injections

ANIMAL	OVARIAN CONDITION	1ST INJECTION	2ND INJECTION
3	Normal	4 hours	7 hours
4	"	0	0
5	"	4 hours	3 hours
6	"	Intermittent estrus 1st part — 1 hour 2nd part — 1 hour	7 hours
8	"	0	0
9	"	2 hours	0
1	Failure of luteinization	0	2 hours
7X	" " "	0	0

DISCUSSION

Although it is clear from the data summarized in the preceding section that the factors which are associated with the failure of estrus in the guinea pig are also encountered in the rat, at least one question presents difficulties which cannot be disposed of so easily. It arises from the fact that a lower than normal sensitivity to estrogen was displayed by all the animals regardless of the ovarian condition. When the guinea pig was being studied there was no opportunity to test the estrogen sensitivity of animals in which deficiencies of hypo-

physeal stimulation were indicated. It was assumed, however, that the sensitivity of such animals was normal and that the failure of heat could be attributed to defective follicular development. The results obtained from the rat have caused us to question this assumption and to ask if an abnormally low sensitivity to estrogen is a part of the syndrome when deficiencies of gonadotropic action are shown. If not, how can the fact be explained that animals in which a deficient gonadotropic action was indicated also displayed a low sensitivity to estrogen?

A tentative explanation is possible if allowance is made for what appears to be an important difference between the rat and guinea pig. This is the point brought out by Boling and Blandau ('39) that the rat, although stimulated to the display of normal heat reactions by the synergistic action of estrogen and progesterone, is nevertheless more sensitive to estrogen. Additional evidence is provided in the first part of this study. Whereas in the normal guinea pig heat has not been seen in the absence of ovulation, in the rat several instances of what appears to be heat without ovulation have been found (Boling, Blandau, Soderwall and Young, '41).

If it is true that the rat is more sensitive to estrogen and that complete follicular development and ovulation are not necessary for the display of heat, some animals of normal sensitivity which show abnormalities of follicular development would come into heat. They would not, however, be discovered in a study such as this because the absence of heat was the basis on which the animals were segregated. On the other hand, when a group of animals in which the heat responses have been normal is classified on the basis of the ovarian condition, abnormalities should be encountered which are identical with or similar to those shown in the last eight animals listed in tables 1 and 2. Our experience has coincided with this expectation. In one of two animals not described elsewhere which were found among those displaying heat behavior, general follicular development was poor and there was evidence for the occasional failure of luteinization. In the other animal

general follicular development was normal, but the occasional failure of luteinization was indicated by the presence of a generation of follicular cysts. Two other animals were found in which no ovarian abnormality was suspected until it was discovered that ovulation had not taken place at the expected time and atresia of the large follicles had begun (Boling, Blandau, Soderwall and Young, '41). Finally the rats studied by Everett ('39) in which abnormal ovarian conditions were found are also known to have mated.

If this explanation for the fact that all the animals displayed a low sensitivity to estrogen is accepted, the two factors that were responsible for the failure of heat in the guinea pig are also associated with the failure of estrus in the rat. These factors were a low sensitivity to estrogen and a deficient hypophyseal stimulation. It seems apparent, however, that the latter does not always prevent the occurrence of heat in the rat because the higher sensitivity to estrogens is such that heat may occur when follicular development is not complete. In the animals we have studied a failure to display heat was shown only when the sensitivity to estrogens was low. This may occur in animals in which the ovarian condition is normal as in the first five animals in tables 1 and 2 or in animals in which the ovarian condition is abnormal as in the last eight animals.

The possibility must be emphasized that this explanation may not be correct. Differences in the threshold to estrogen action have long been known (Wiesner and Mirskaia, '30; Hemmingsen, '33; Hemmingsen and Krarup, '37), but factors which might influence sensitivity have only recently been considered (Young, '41) and are only now being investigated. When it is known what factors are responsible for the changes which occur during development (Wilson and Young, '40) and the differences shown by adults, a reinterpretation of our results may be necessary. At the moment, however, the low sensitivity of animals in which the ovarian condition appears to be normal, and the discovery of animals in which heat is displayed despite an obviously abnormal gonadotropic action

would seem to exclude the possibility that a hypophyseal deficiency was responsible for the low sensitivity found in the animals considered above.

SUMMARY AND CONCLUSIONS

A group of female albino rats which displayed heat only irregularly if at all has been studied in an effort to ascertain if deficiencies of hypophyseal stimulation and low sensitivity to estrogen are responsible for failure to display sexual receptivity. Information with respect to the character of hypophyseal stimulation was obtained from a study of the ovarian condition and the sensitivity to estrogen was determined by means of injections of different quantities of this hormone followed by progesterone.

1. In eleven of twenty-one animals the ovarian condition appeared to be normal. In the remaining ten a variety of ovarian abnormalities are seen, all of which are believed to indicate a deficiency of hypophyseal stimulation. These abnormalities consist of a poor general follicular development accompanied by an atresia of most of the small follicles, an apparent failure of luteinization after the follicles reach the stage at which the preovulatory swelling ordinarily begins, or both.

2. The ovarian abnormalities encountered were not necessarily permanent. Variations occur, sometimes in the direction of normality and sometimes in the direction of greater abnormality.

3. All the rats displayed a low sensitivity to estrogen. Inasmuch as other rats were found in which heat occurred despite the failure of ovulation and normal follicular development, it is concluded that in the animals studied in this investigation the basic factor in the failure of heat was the low sensitivity to estrogen.

4. The same factors that sometimes account for the failure of heat in the guinea pig are present in the rat, but deficiencies of hypophyseal stimulation are postulated to be involved less frequently in the rat because the sensitivity to estrogen ap-

pears to be such that heat is less dependent on normal follicular development.

5. Additional evidence is provided for the importance of progesterone in the induction of heat in the rat.

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PLATE 1

EXPLANATION OF FIGURES

1 Section through second ovary removed from animal 150A which is not known to have been in heat prior to ovariectomy. Two generations of corpora lutea and small normal appearing follicles can be seen. $\times 20$.

2 Section through second ovary removed from animal 152 which is not known to have been in heat prior to ovariectomy. No corpora lutea are present. Two cystic follicles of an older generation and three atretic follicles of a more recent generation can be seen. $\times 20$.

3 Section through first ovary removed from animal 100 which is not known to have been in heat prior to ovariectomy. For comparison with figure 4. Cystic follicles of two generations are present and the edges of two corpora lutea are visible. General follicular development is poor. $\times 20$.

4 Section through second ovary removed from animal 100. The corpora lutea of two generations and growing follicles are evidences of the better general follicular development. $\times 20$.



AN EXPERIMENTAL ANALYSIS OF THE ALTMANN TECHNIC OF FREEZING-DRYING ¹

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ONE PLATE (EIGHT FIGURES)

The intelligent application of the Altmann technic of freezing-drying fixation to a specific cytological problem demands the evaluation and integration of the various factors in the method which might individually modify the ultimate quality of cellular preservation. The determination of factors that might affect the end result of the process had already begun at the time Gersh ('32) introduced the modern form of the technic. Gersh pointed out the effect on the final fixation of (1) the size of the sample of tissue frozen, (2) the relation of tissue volume to surface area, and (3) the water content of the tissue used. Scott ('33) noted (4) that the rate of freezing as determined by the heat conductivity of the freezing medium influenced the end result, as did (5) the temperature of dehydration. Hoerr ('36) in a review of the method added to these (6) the effects of changes in the rate of dehydration as influenced by the degree of vacuum employed and (7) the effect of miscellaneous factors involved in the handling of the dried material.

This paper presents results of an experimental evaluation of these and of other determinable factors for the role that each plays in adequate preservation of cellular constituents.

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MATERIALS AND METHODS

The results to be reported are based for the most part on guinea pig liver, pancreas, kidney, epididymis, duodenum, testis, adrenal, and spinal ganglion, and rabbit spinal cord and spinal ganglion. The pieces of test tissues ranged in size from half of a guinea pig thoracic dorsal root ganglion to whole guinea pig testes, kidneys, and spleens. To facilitate handling the individual pieces without losing track of them or of what had been done to them, the tissues were isolated in tiny numbered balloon silk bags. This did not appear to slow the process of dehydration.

In order to study the effect of increasing the ratio of surface area to tissue volume, tissues were stretched on cardboard, teased out, and even smeared on cover slips prior to freezing.

Freezing was brought about in (1) isopentane chilled in liquid nitrogen to temperatures ranging from -60 to $-195^{\circ}\text{C}.$, (2) directly in liquid nitrogen, (3) in liquid nitrogen after enclosing the test tissue in a thin covering layer of muscle or liver, and (4) on the surface of a brass block chilled in liquid nitrogen.

The latter method, freezing on a chilled brass block, was devised in an attempt (1) to obtain more rapid cooling of the tissues by the use of a substance having much higher capacity for heat conduction than either liquid nitrogen or isopentane, and (2) to establish definitely the direction of freezing through the tissue so that the progress of freezing might be correlated with the structural changes observed. The brass mounting block from a rotary microtome was used. Its concentric ridges made possible permanent identification of the side of the tissue with which it had been in contact.

To study the effect of freezing on the subsequent chemical fixation, tissues were frozen as usual and then thawed in chemical fixing solutions at temperatures of from 0° to $37^{\circ}\text{C}.$ Absolute alcohol, Zenker-formol, and a wide variety of the commonly employed fixatives were used in this manner. Dehydration, clearing, and embedding followed this procedure which will be referred to as "freezing-thawing" fixation.

In an effort to isolate the effect of freezing from the effect of subsequent dehydration in vacuo, tissues were frozen and placed in organic solutions at temperatures of from -40° to -78°C . The only solvent used at the lower temperature was Methyl Cellusolve (National Carbon Co.); Methyl Cellusolve, absolute ethyl alcohol, anhydrous diethyl ether, and anhydrous chloroform were used at the higher temperatures. This procedure will be called "freezing-substitution".

Vacuum dehydration in the freezing-drying technic was carried out at temperatures of from -30° to -50°C . After dehydration, the tissues were either removed directly to a desiccator or allowed to rise to room temperature before being transferred. Free hand sections of these tissues were examined in the manner suggested by Hoerr ('36), using 2 mm. and 3 mm. apochromatic objectives, polarized light, the cardioid dark field condenser, and vertical illumination by the Ultropak microscope.

Tissues for routine examination were denatured for at least a week in redistilled absolute alcohol. Denaturation was attempted also by heat and the use of chemical fixing agents such as osmic acid, potassium dichromate and numerous tissue fixing mixtures. These procedures appeared to have a very limited use. Embedding was done in paraffin, in nitrocellulose, or by the methyl benzoate celloidin and paraffin technic.

OBSERVATIONS

1. Effect of the size of the piece of tissue frozen

It was observed, as was noted by Hoerr ('36), that even relatively large pieces of tissue might be frozen in chilled isopentane without cracking, with a final appearance that compared favorably under the lower powers of the microscope with similar tissues preserved in such a mixture as Bouin's fluid (figs. 1 and 6). This was true if general histologic stains were used. With more complex mixtures of dyes, such as Heidenhain's modification of Mallory's connective tissue stain (the Azan stain), even low powers of the microscope revealed

striking variations in the response of different layers of the tissue to the dyes. Guinea pig liver, for example, showed three rather sharply demarcated zones of staining. The outer zone took the stain in much the same manner as a well-fixed Zenker-formol preparation. Inside this zone was another in which the cells took the azocarmine and orange G rather too strongly. Next to this was yet another zone which stained in a nearly normal fashion, possibly taking the aniline blue a little too strongly. There then appeared in succession one or more repetitions of the last two zones, the difference in staining between adjacent layers being not so marked as in the outer regions. With the regular Mallory connective tissue stain, the irregularities of staining in laminae appeared even more marked.

Examination of this material under a 2 mm. oil immersion apochromatic objective revealed that the outer "normally" stained zone described above presented a quality of fixation which compared very favorably with the best obtainable chemical fixation of guinea pig liver. The homogeneity of the nuclear juice and of the cytoplasmic background, the distinctness and uniformity of the mitochondria, the perfectly spherical fat vacuoles, the highly refractile cell membrane, the clear-cut staining of the walls of the bile capillaries, the smoothness of the connective tissue fibers, all pointed to the fact that here fixation was equal to or superior to the best chemical fixation yet devised. It may be noted that there existed on the very surface of the block a zone, usually only one cell thick, in which the cells tended to be darkly stained. This was true of all tissues and all stains tried. It appeared to be characteristic of frozen-dried material. No reason for this has been found.

The second major zone of tissue presented a marked contrast to that just described. Throughout this zone the cells showed serious artefacts of freezing. The ice crystals produced in the cytoplasm had disrupted the fat vacuoles and deposited proteins along their edges so that mitochondria were

not identifiable. The nuclei were badly reticulated by ice crystals and the connective tissues were hardly recognizable.

The next major area, laminated in staining density, was more or less uniform in quality of fixation, the quality falling off toward the center of the larger blocks. In this area the connective tissues were poorly preserved. A pattern of large ice crystal artefacts broke up the connective tissue nuclei and fibers generally. Blood vessels containing blood showed a reticulate structure in the lumen in contrast to the uniform preservation of the erythrocytes and white cells in the first described outer zone. The preservation of epithelial cells of the area represented a compromise between the excellent outer and the very poor second zones described above. The artefacts were small enough so that nuclear detail was preserved fairly well, although a definite disruption by ice crystals was uniformly apparent. Cytoplasmic detail was quite well preserved, but here too, a fine reticulation was generally present. The differences which appeared between the secondary zones within this general area indicated that preservation was poorer in the cells taking the azocarmine and orange G too strongly.

The same type of zoning occurred in other tissues where the mass was relatively great and the organ more or less compact. Guinea pig suprarenal gland, after isopentane freezing, usually showed excellent preservation of the zona glomerulosa and of the outer part of the fasciculata. The inner part of the fasciculata usually contained a narrow zone in which nuclear ice crystals were very large. The lipin droplets in this area were less regularly round and smooth; the nuclei were badly broken by ice artefacts. Deeper in the fasciculata, reticularis, and medulla the quality of fixation was again quite good, though still inferior to that in the outermost zone. That this phenomenon was not due to inherent differences between the parts of the gland was shown by the fact that wherever a little of the surface of the organ was covered by adherent connective tissue, the zone of poor fixation shifted toward the capsule and came to lie in the zona glomerulosa or even outside the organ in the connective tissue covering.

Small bits of these tissues or of others did not show all the zones described after isopentane freezing. If small enough, the fixation might be excellent throughout. No absolute limits can be stated for a maximum size in which this type of preservation occurs exclusively since the thickness of the outer zone varies over rather wide limits in different tissues. Reasons for the variation in the response of different tissues to freezing will be discussed later.

Tissues frozen by compression between the chilled brass block and a nonconductor such as paper or cork substantiated the observations recorded above. Here the heat was conducted away from one side of the tissue only and therefore freezing had perforce progressed through the entire sample from that side. The picture obtained by this method corresponded to that resulting from isopentane freezing with the exception that the general quality was lower throughout (fig. 1). From a fairly good surface zone (fig. 2), a zone (fig. 3) was delimited which was so disrupted that, were it alone, the tissue might not have been recognizable. Beyond this again appeared fixation (fig. 4) that at least permitted recognition of the tissue although it was poorly preserved in comparison with the surface layer.

2. The effect of the relation of tissue volume to surface area

Certain tissues, because of their peculiar structure, have a large surface area in relation to their volume. Such are the following guinea pig tissues: pancreas, duodenum which has been slit open, gastric mucous membrane, and, to a lesser extent, the epididymis. It is possible to make very thin slices of kidney and liver, also, and to increase artificially the surface of testis and pancreas by teasing and even by smearing them onto cover slips before freezing. When such tissues were frozen and dried, the quality of the fixation was superior to that of the same tissues frozen in compact masses. In most cases the fixation partook of the characteristics of the outer zone previously described, but in the testis the zone of the

best freezing-drying extended only a few cells from the surface (fig. 8).

Materials prepared by the "freezing-substitution" method previously described, showed a quality of fixation in most respects exactly comparable with frozen-dried tissues. The zoning of ice crystal artefacts and the very presence of these artefacts indicated that the freezing proper and not the dehydration or any subsequent step in the freezing-drying process was responsible for the characteristics of the material described above. Fixation by the "freezing-substitution" method is essentially a chemical one, however. By this method proteins are precipitated in a denatured state and react to stains in a fashion characteristic of chemically fixed tissues.

3. Effect of water content of the tissue frozen

It has been uniformly true that the tissues which are most fluid are the ones in which the most severe damage by ice crystals has been observed. Tissues in which this severe disruption has been found more than a very short distance from the surface are: loose connective tissue, nervous tissue, testis, and portions of the blood vascular system such as lymph nodes, spleen, bone marrow, and blood itself.

Attempts to reduce the amount of water in tissues by depriving the animals of food and water prior to killing (Bensley and Gersh, '33 a) have met with only partial success. The best results following this procedure have been in tissues where freezing-drying is already most successful in undehydrated animals. Little, if any, improvement has followed its use in preparation of testis, spinal cord, and brain—tissues that are the most difficult to preserve satisfactorily by freezing-drying.

4. Effect of the rate of freezing

In an attempt to discover whether there existed an optimum rate of freezing other than the fastest rate obtainable, a series of freezings was carried out at temperatures of $-60^{\circ}\text{C}.$, $-95^{\circ}\text{C}.$, $-120^{\circ}\text{C}.$, $-140^{\circ}\text{C}.$, $-165^{\circ}\text{C}.$, $-184^{\circ}\text{C}.$, and

— 195°C., using guinea pig liver as the test tissue. The typical appearance of guinea pig liver frozen in isopentane at the lowest temperature before it solidifies has already been described. In this series of experiments it was found that the only result of the elevation of the freezing temperature could be interpreted as a gradual shifting of the zones of fixation toward the outside of the block. Thus, at the highest temperature, the fixation of the tissue as a whole was almost identical with that of the inner zone of the tissue previously described. Intermediate freezing temperatures of — 100°C. to — 140°C. showed preservation in which the surface freezing was comparable with that of the intermediate very poorly fixed zone of the material frozen at — 195°C.

5. Effect of the temperature of dehydration

In general, tissues dehydrated at — 40 °C. exhibited a more uniform quality of preservation than those dehydrated at — 30°C. or above. On the other hand, for purposes of cellular morphology, at least, there did not appear to be any choice between — 40°C. and — 50°C. The greatly increased time necessary for dehydration at the lower temperature is an argument against its general use.

6. Effect of the rate of dehydration

At lower temperatures of dehydration, the use of a triple stage mercury diffusion pump throughout the period of dehydration was not found to cause disruption of cells by too rapid removal of water vapor as was thought to be the case (Hoerr, '36) at temperatures of — 25°C. to — 30°C.

7. Effect of miscellaneous factors in handling dried tissue

The rapidity of handling dried tissues was of extreme importance in preventing them from taking up atmospheric moisture. Particularly was this true of fatty tissue such as frequently surrounds the pancreas. Pieces of dried pancreas were found to take up water so rapidly from a humid atmo-

sphere that before free hand sections could be cut the entire block was soft and spongy. To avoid the further complication of atmospheric moisture condensing on cool blocks, it was desirable to allow the blocks to come to room temperature before exposing them to the air at all.

Since the freezing-drying process does not completely remove water from tissues, it was thought cells might be caused to shrink in the process of vacuum embedding. Denaturation of the tissues in redistilled absolute alcohol for a week or more left the proteins in such condition as to permit shrinkage in the process of paraffin embedding following cedarwood oil clearing. The best preservation of general histological detail followed embedding in celloidin without the use of heat. This method of preparation has, for the first time in the author's experience with frozen-dried tissues, preserved the characteristic differences between collagenous and reticular connective tissue fibers in the loose interstitial connective tissue of the epididymis. This technic has also been successful in preventing shrinkage of spermatocytes in active mitosis (fig. 8), something which had hardly been seen, except for early pro-phases, in frozen-dried testis embedded in paraffin.

DISCUSSION

Four factors have been described which are believed to modify the quality of cytological fixation by the Altmann technic. A study of these factors might be expected to throw some light on the reasons why Bensley and Gersh ('33) found that freezing was most satisfactory in large pieces of tissue and in tissues made larger and protected by an added covering of other tissue; as well as why now our best cellular preservation results from the use of the very outside of small bits of tissue having a relatively large surface area.

Bensley and Gersh ('33, p. 210) state that: "In the original experiments the cytological fixation was least satisfactory at the surface of the blocks, due to the formation of ice crystals."

Scott ('33) explained this improved fixation beneath the surface on the basis of the direct observations by Chambers and Hale ('32) of freezing of muscle fibers. These workers observed that preceding freezing there was a dehydration due to the freezing out of "free" water and the consequent elevation of the salt concentration within plasma membrane. Scott further suggested that equilibrium of temperature was probably not reached for some time after a piece of tissue was immersed into liquid air and that free water might be frozen out first in this case as it was in the much slower process described by Chambers and Hale. His basis for this contention was that the tissue was not in contact with liquid air but with air in the gaseous form, which is a rather poor conductor of heat. The superior results obtained by freezing in a liquid such as ethyl alcohol, pentane, or isopentane are easily understood if the rate of freezing is a major factor, for at ordinary temperatures these liquids conduct heat at nearly eight times the rate of nitrogen gas. Attempts to speed freezing by the use of a relatively great mass of brass, the heat conductivity of which is nearly 600 times that of ethyl alcohol, proved unfruitful because it was impossible to change the surface of the block that was in contact with the tissue. Fluid baths have the further advantage of contact with all surfaces of the tissue simultaneously.

Satisfactory preservation by freezing-drying of large pieces of tissue appeared to be limited less by the heat conductivity of the freezing medium than by that of the already frozen surface of the tissue, a factor which can hardly be controlled. So long as the fluid bath had a higher rate of conductivity than frozen tissue had, the use of different fluids for freezing media could affect the preservation only within a thin surface layer. It has been observed that the organs most satisfactorily preserved are those with a loose lobular arrangement. This may be correlated with the fact that the connective tissue partitions of these organs are bulky and probably freeze readily by the propagation of ice crystals from the surface inward. The ice thus formed is a much better conductor of heat than is liquid

water, and allows a rapid withdrawal of heat from the parenchymatous portion of the organ.

The experiments in which freezing was produced over a wide range of temperatures showed that the effect of lowering the freezing temperature did not bear a constant relation to the adequacy of the resulting fixation. These experiments indicated that freezing preservation was best when the temperature of the isopentane was as low as possible, certainly below $-160^{\circ}\text{C}.$, and that if this temperature or lower was not obtained, the next best result could be expected at such a temperature that the zone of very large ice crystals was not produced, that is, about $-60^{\circ}\text{C}.$ Similar results were at times obtained directly from liquid air or liquid nitrogen.

The zones produced within the area of "secondary" freezing cannot yet be explained satisfactorily. The morphological and staining reaction differences between adjacent zones must be interpreted as evidence of physical and chemical alterations of the tissue in the freezing process. Many factors complicate the interpretation of this area. One of these is the possibility of development of considerable pressure inside the block as the surface freezes. The expansion ordinarily accompanying the formation of ice crystals is prohibited by the surface shell of relatively inelastic frozen tissue. The resulting pressure may either crack the block, as it frequently does, or may cause a further slowing of the internal freezing through liberation of heat of compression and through depression of the freezing point by pressure, both of which are well recognized phenomena.

Since the rate of freezing of the interior of the block is determined by the rate of heat conduction by the already frozen shell of tissue, and is possibly slowed by the increased pressure, it seems probable that the quality of the preservation in this area may depend upon the freezing out of water and the concentration of salts within the cells, as was found by Chambers and Hale to be the case in the slow freezing of tissues. The fact that in most tissues the ice crystal artefacts in the intercellular spaces within the block were much larger

than the artefacts within the cells of the same region further supports this view. To be sure, a part of this may be due to original differences in water content of the cells and the intercellular spaces. The fact that the cells in this region differed so greatly in their freezing artefacts, although not greatly in water content, leads us to believe that within some cells the freezing was initiated rather soon by the appearance of an ice crystal nucleus, while in others the lack of the appearance of such a nucleus permitted the same type of diffusion of water out of the cell to the intercellular spaces as was noted in the work of Chambers and Hale. Kistler ('36) pointed out that there is adequate reason to believe that in biological systems the water is in such small capillary droplets that much of it may be cooled to very low temperatures without freezing, since as the temperature is reduced, the activity of the water decreases as does also the tendency toward appearance of nuclei of crystallization. Water in cells in which no crystal nuclei appear can freeze only by diffusion out of the cell onto an already frozen surface. The intercellular spaces, which are continuous over relatively great distances, provide such a freezing surface.

If these changes in the distribution of intracellular fluid take place within blocks of tissue, it may be expected that changes in the intracellular distribution of highly soluble and highly diffusible salts accompany them. Studies on the precise distribution of such salts, then, appear best confined to the surface zone of tissues that are rapidly frozen at the lowest possible temperature in a fluid from which gas is not evolved during the freezing process.

Concerning the effect of water content of tissues on the success with which they may be frozen-dried, it is of interest to note the amount of change in weight of animal tissues through dehydration by acute thirst, presumably mainly by loss of water. Kudo ('21) noted in a series of adult albino rats that acute thirst produced a gross weight loss of 36.1%. Tissues which have been most successfully frozen following dehydration of the animals, with the weight loss by thirst of

the same tissues from Kudo's series, are: pancreas (53.1%), liver (37%), stomach and intestines (36.4%), epididymides (30.0%) and kidney (23.8%). On the other hand, the failure of dehydration to improve the fixation of testes is explained by the fact that acute thirst causes loss of only 15.1% of their weight. Failure to freeze central nervous system tissues more satisfactorily following dehydration of the animal is correlated with the fact that in acute thirst the brain increases in weight by 0.12% and the spinal cord by 1.80% (Kudo, '21).

CONCLUSIONS

These studies on the process of freezing and on other factors controlling fixation by the Altmann method lead us to the following conclusions:

1. The nature of the coarsest reticulation in tissues prepared by the Altmann technic is due to ice crystals and not to the treatment of the tissue after freezing. This was established by (a) the larger size of the artefacts in tissues with a high water content, (b) the absence of such artefacts in tissues prepared by the freezing-thawing method, and (c) the presence of such artefacts in tissues prepared by the freezing-substitution method.

2. The degree of this reticulation is influenced by: (a) the rate of freezing, (b) the water content of the tissue originally and as reduced by dehydration of the animal prior to killing, and (c) changes that occur in free water and salt concentration during the process of freezing.

3. Preservation of cytological details in the outer layer of tissues frozen in chilled isopentane at -195°C . surpasses in excellence chemically-fixed tissue. The completeness of the cellular constituents in a form unmodified except for drying makes this the fixation of first choice for purposes of cellular chemistry.

4. The freezing fixation within larger blocks of tissue is inferior to that of the surface zone, from which it is separated by a layer of very poorly preserved tissue. This inner zone may be, nevertheless, superior to chemical fixation for general

cytological and histochemical studies. The zone is not suited to precise chemical studies since it is probable that, at least in part, the general quality of preservation is due to changes in water and salt concentrations during the freezing process.

5. Denaturation of proteins of frozen-dried tissue preparatory to morphological studies is best assured by the treatment of the blocks for at least a week in redistilled absolute ethyl alcohol. This should precede clearing and embedding for optimum histological preparations.

6. Since tissues are subject to modification by heat even after alcohol denaturation, embedding in celloidin is recommended as a routine procedure.

It is with pleasure and gratitude that I acknowledge my indebtedness to Professor R. R. Bensley for kindly criticism and helpful suggestions and to Doctor N. L. Hoerr, under whose direction this work was carried out, for continuous encouragement and cooperation. To all the members of the Department of Anatomy I am grateful for many courtesies shown me during the period of this investigation.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

1 Guinea pig liver frozen by contact with a chilled brass plate and dehydrated in vacuo. The surface to the left was against the brass; that to the right against cork at room temperature. Dried block denatured in alcohol. Paraffin embedded. Stained with Hansen's chrome-alum-hematoxylin and eosin. Photomicrograph across the width of an entire section. $\times 41$.

2 Portion of a high power field near the left edge of the section shown in figure 1. $\times 370$.

3 Portion of a field near the center of the same section. $\times 370$.

4 Portion of a field near the right edge of the same section. $\times 370$.

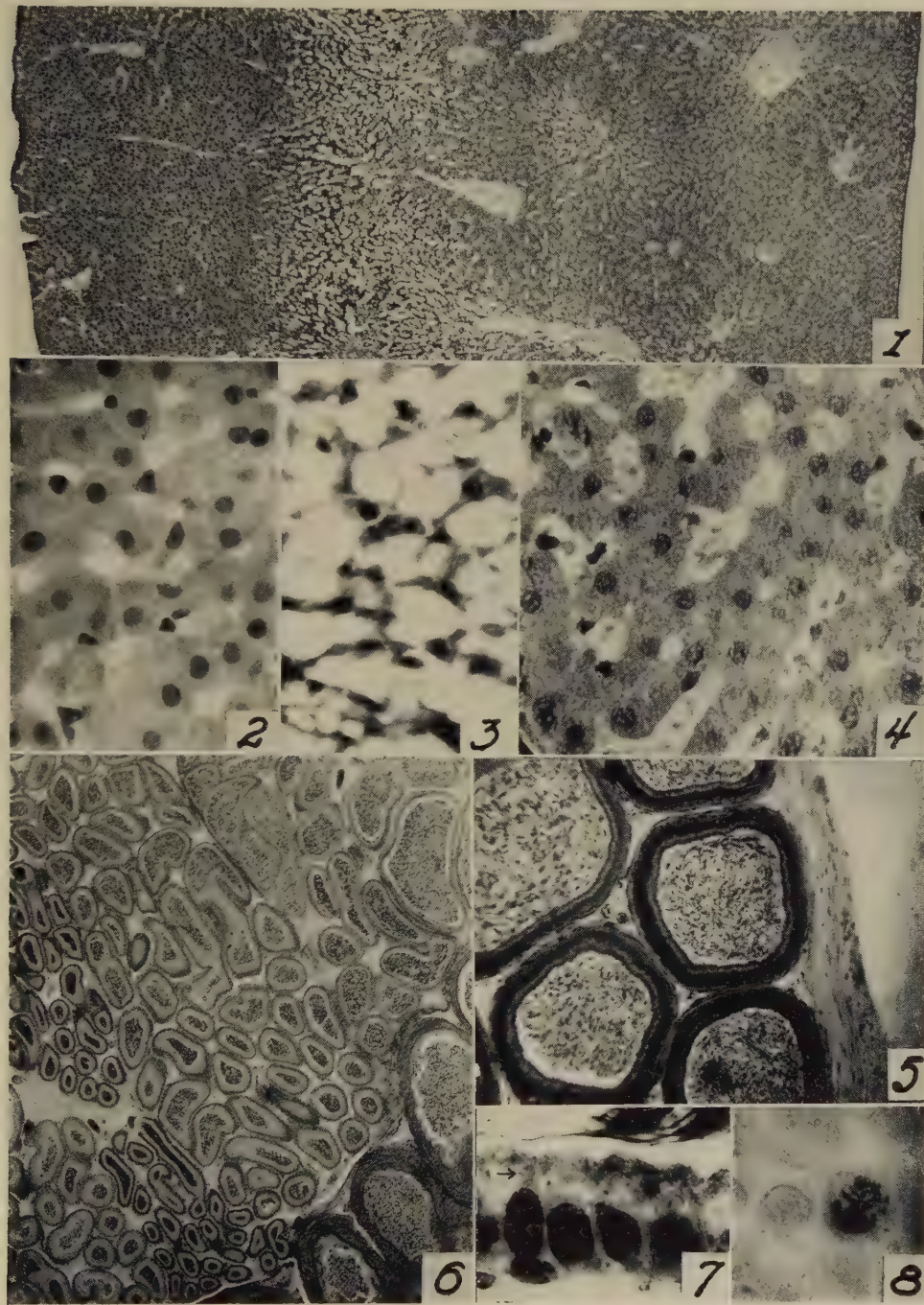
5 to 7 Guinea pig epididymis. Frozen in chilled isopentane and dried in vacuo at -40°C . Alcohol denatured. Celloidin embedded. Stained as indicated below. All are photomicrographs.

5 Stained with iron hematoxylin. This field shows the freedom from shrinkage artefacts and the general high quality of fixation of the surface region of tissues by the Altmann technic. $\times 140$.

6 Mallory-Azan stained. This very low power indicates how well relatively large pieces of loosely arranged tissues may be preserved by freezing-drying. $\times 20$.

7 Stained with iron hematoxylin. Oil immersion. Shows the quality of cellular preservation. Note central body and centrioles near end of arrow. $\times 1000$.

8 Guinea pig testis showing the preservation of a cell in mitosis near the surface of the frozen tissue. Such preservation extends at best only a few cells deep in testis. Celloidin section. Iron-hematoxylin. $\times 1000$.



A CONTRIBUTION TO THE TECHNIQUE OF DETERMINING THE NUMBER OF NERVE CELLS PER VOLUME UNIT OF TISSUE

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FOUR FIGURES

In the capacity of expert,¹ I have had to define the view I take of the v. Economo nerve cell figures for the human cortex. In my opinion, it is necessary to make it clear that these figures cannot be accepted even as approximately accurate.

Why cannot the v. Economo nerve cell figures be accepted as even approximately accurate?

These figures refer to the number of cells per volume unit of the human cortex, and the calculations are carried out on photomicrographs, enlarged 100 times. When taking my doctor's degree, 20 years ago, I even then countered a remark from one of my opponents by saying that, though it was admittedly conceivable in theory, in practice it was hardly possible to count on photomicrographs the total number of nerve cells in ganglia or nerve fibers in cross section from nerve roots. I have every reason for keeping to this contention. During the past 20 years, however, several investigators in this field have made use of the photographic method for this purpose, but it is easy to show that in so doing they misled themselves in so far as their calculations actually referred to the total number of these elements. V. Economo is one of

¹ The expert examination dealt with a thesis (Lingren, '40) which was submitted in a competition for Svenska Läkaresällskapets Alvarenga prize for 1940.

these investigators. Even after Lindgren in 1940 had pointed out a fatal error of calculation by v. Economo and the values were adjusted accordingly, these v. Economo values for the nerve cell number in the human cortex are to a high degree misleading and impaired by considerable methodological errors. The most outstanding of these are due to the fact (a) that not all the ganglion cells in the third dimension of the section have been distinctly reproduced on the photomicrographs. (b) That it is impossible to differentiate on the v. Economo photomicrographs (magnification only 100:1) between small nerve cells and glia cells. (c) That no consideration is paid to the fact that one and the same nerve cell can fall within more than one section.

a. By means of low magnification (100:1), suitable optics and axial rays, v. Economo has succeeded in attaining a considerable depth of focus in his photomicrographs. It follows from the nature of the experiment, however, that many nerve cells cannot appear distinctly on the photomicrographs—some do not appear at all—being more or less completely covered by cells lying above them. Anyone who has gone a little more deeply into these matters knows that nerve cells inside the cortex sometimes lie so near to one another that the strongest resolving microscope is needed to make them appear as separate cells. Even this fact is an indication of how impossible it is to count the total number of nerve cells on photomicrographs.

b. An even more discordant fact is that the low magnification (100:1), combined with the intense staining of the cells and the powerful photographic exposing makes it impossible to differentiate between small nerve cells and glia cells on the photomicrographs of v. Economo. Many such small nerve cells are present in the cortex, but their numbers in the different nerve cell layers vary. This means that the unreliable and faulty results to which this methodological source of error leads, affect the nerve cell layers to different degrees.

c. In drawing up the v. Economo figures for the number of nerve cells per volume unit of cortex, no consideration has been paid to the fact that one and the same nerve cell in section from the cortex can fall within more than one section.

The authors (v. Economo and Koskinas) write *re* this:

Wir haben dagegen alle Nervenzellen, die innerhalb eines Quadrates von 1,0 mm Seitenlänge (1 cm unserer Tafeln) fallen immer mitgezählt; dadurch sind unsere Zahlen absolut genommen etwas zu hoch, da auch die in die beiden Schnittflächen, in die vordere sowohl als in die hintere fallenden Zellen und Zellstücke mitgezählt sind. Der Fehler, der hierbei begangen wird, beträgt bei 25 μ Schnittdicke für die Zellgrössen bis herab zu 12 μ Zellbreite ein Plus von ungefähr 33% für mittlere und kleine Grössen bis zu 6 μ Breitendurchmesser ein Plus von 25% und für Zellen unter 6 μ ein Plus von ungefähr 20—15%; dieses Plus müsste also von den in unseren Tabellen im 14. Kapitel angegebenen Zellzahlen abgezogen werden, wenn man *absolut richtige Zahlen des Zellreichtums*² vor sich haben will, eine Berechnung die ja da Tabelle 5 die Zellgrössen und Tabelle 6 die Zellzahl jeder einzelnen Gegend gibt, sehr leicht durchzuführen ist. Uns kommt es jedoch, ausser bei dem späteren Versuch die absolute Zellenzahl der Totalität des Grosshirns annähernd zu bestimmen, etc.

As will be shown below in more detail, this information as to the reduction figures is far too inexact to be anything like a correct guide. Consequently one must question the accuracy of "absolut richtige Zahlen" derived by means of such reductions, even if the figures to be reduced were otherwise correct. And the figures in question are, for reasons given above, anything but correct. The inexactitude of the figures is, moreover, probably greater in the layers with the smaller nerve cells than where mainly larger nerve cells are to be found.

² The italics are mine.

How is one to set about a determination of the number of nerve cells per volume unit of tissue, so as to reach the most accurate values possible?

Under no circumstances is it possible to obtain the "absolutely correct values" of v. Economo and Koskinas (supra) for the number of nerve cells in the cortex, but instead of the absolutely incorrect v. Economo figures, values can be reached which as far as possible approach the right ones.

The nerve cells in Nissl stained sections of a fixed depth and made at right angles to the longitudinal course of the gyri, are counted under the microscope. The latter should have a precision-built cross-table or mechanical stage, which makes possible an exact adjustment of the preparation. By this means, the part which is to be examined can be covered systematically without any area being missed or being counted twice. While counting, an oil immersion should if possible be used, and also a square ocular micrometer which projects small squares into the field of the microscope, the length of whose edges in the optic plane is exactly known in the enlargement used. In this way, the squared field can at the same time act as a micrometer also.

While the optic plane is made to move between the two cut surfaces of the section with the help of the micrometer screw of the fine adjustment, all those nerve cells are counted whose nuclei wholly or to some extent fall within the section. To avoid counting the same nucleus more than once, the same process is followed as in the counting of blood corpuscles. Cell after cell is recorded in a table, the columns of which represent nucleus diameters, measured in whole and half μ respectively, for example:

Nucleus diam. in μ	—	1	1.5	2	2.5	3	3.5	4	...	10	10.5	etc.
Number of nerve cells												

The nuclear diameters are of course measured when the optic plane coincides with the greatest sectional area of the nucleus. Should the nuclei not be circular, but inclined to an elliptical form in the plane, a diameter of $2\sqrt{Rr}$ is taken,

where R and r are half of the large and half of the small diameter respectively. The arithmetical mean of the two diameters gives a rather too high value, but this source of error will not be particularly great in any case. And the fact that the nuclear form which deviates from the spherical does not always approach the elliptical, means that it is impossible to lay down any fixed procedure; here the method of procedure must be adapted to suit each case. In this way squared field after squared field is used to that extent to which it may be necessary to even up possible variations in cell number and cell position.

If it is a question of determining the cell number in the different laminae of the cortex, the number of nerve cells in all squared fields in transverse section from the cortex must be counted and systematically entered in the table mentioned above. With the help of a photomicrograph, the counted squared fields can then be referred to the appropriate laminae. Here it would not be out of place first to determine, with the help of the photomicrograph, how many squared fields belong to this or that layer respectively. In so doing, measurements are made from the surface of the cortex towards the medullary layer.

In determining the number of nerve cells in a ganglion, for example, the latter is laid out in a consecutive series of thin (e.g. 5μ) sections, which after suitable staining are submitted to a systematic nerve cell counting, in the same way—as far as may be found suitable—as has been given above. It is possible to determine by special tests how close the sections should be in the series where the cells are to be counted. (In addition to this, a reconstruction, in paper of a uniform thickness, might be very useful here in facilitating the work without jeopardizing the exactness of the results.)

The values for the nerve cell numbers obtained by the process given are too high, because one and the same nerve cell can fall within more than one section. It is easy to see that the smaller the counted bodies are in relation to the depth of the section, the less will the value of the cell number

In a manner analogous with that in the foregoing case, we get

$$\frac{2a-d}{a} \cdot \frac{1}{2} + \frac{d-a}{a} \cdot \frac{1}{3} = \frac{6a-3d+2d-2a}{6a} = \frac{4a-d}{6a}$$

If the displacement of the body is continued in the next section, we get the same results again. The number of cell nuclei or spherical bodies ($=n$) counted in the section is thus to be multiplied by this formula to give the true number. The formula in alternative (2) thus becomes:

$$\frac{4a-d}{6a} n$$

In the next alternative we say:

$$2a < d \leq 3a \quad (3)$$

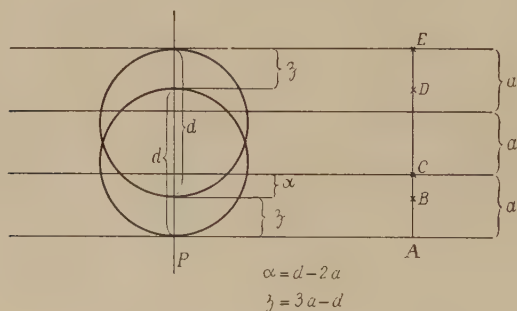


Fig. 3 Three $a \mu$ deep sections.

In a manner analogous with that for alternatives (1) and (2), we get:

$$\frac{3a-d}{a} \cdot \frac{1}{3} + \frac{d-2a}{a} \cdot \frac{1}{4} = \frac{12a-4d+3d-6a}{12a} = \frac{6a-d}{12a}$$

The formula in alternative (3) will consequently be:

$$\frac{6a-d}{12a} n$$

One could continue thus or—which would be better—try to give the formula a general application by letting the d of

the body extend over an m number of sections. The alternative can then be written:

$$(m-1)a < d \leq ma \quad (4)$$

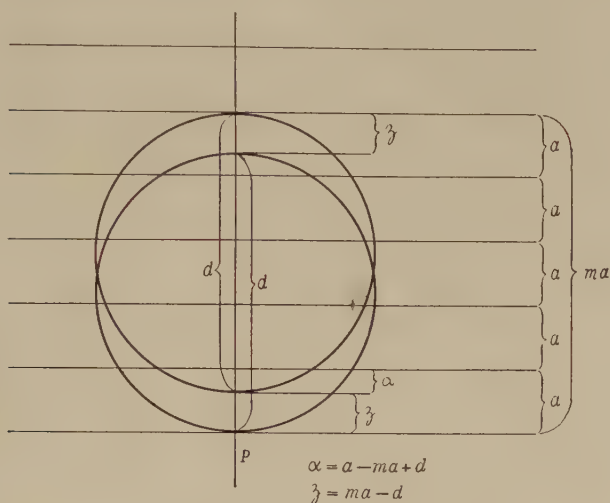


Fig. 4 m a μ deep sections.

In a manner analogous with the foregoing, we get from this:

$$\begin{aligned} \frac{1}{m} \cdot \frac{ma-d}{a} + \frac{1}{m+1} \cdot \frac{a-ma+d}{a} &= \frac{(m+1)(ma-d) + ma - m^2a + md}{ma(m+1)} \\ &= \frac{m^2a - md + ma - d + ma - m^2a + md}{ma(m+1)} = \frac{2ma-d}{ma(m+1)} \end{aligned}$$

In a way which was indicated in a previous alternative, the appearance of the formula is not changed if the center of the nucleus continues to be moved upwards and downwards respectively along the line P . The formula for the general alternative (4) will consequently be:

$$\frac{2ma-d}{ma(m+1)} n$$

This formula applies to all spherical bodies of a certain order of magnitude, provided they are evenly distributed throughout the depth of the section.

If it is a question of determining the number of bodies which are of different sizes, the bodies should be grouped according to size and then the number should be reduced for each separate group, with the help of the formula.

The validity of the formula is not affected even should the spherical bodies be unevenly distributed in the spread of the section. On the other hand, a fairly regular distribution is expected in the depth of the section (i.e. in a perpendicular direction), though an irregular distribution even in this direction can be ignored if "bodies" are counted over a sufficiently large area.

It is also possible to compensate to a large extent for deviation from the spherical form in the bodies. The shape of the nucleus not infrequently tends towards the ellipsoid. In thin sections, only two of the three axes of the ellipsoid can as a rule be met with. The diameter in the circle surface corresponding to the ellipse surface in the section in which the axes can be measured direct, is $2\sqrt{Rr}$, where R and r are half the lengths of the ellipse's long and short axis. If it is not a rotation ellipsoid, but instead one where all the three axes have different lengths, the third axis comes into the calculation all the same: it is included by virtue of the body's presence and the fact that it is counted in a more or less large number of the adjacent sections. Why we are justified in deducing the lengths of the axes of the ellipse to the diameter of a circle with a corresponding surface is because the short and the long ellipse axis equally often chances to fall in all directions within the section or sections.

It should of course be added to what has already been said that this method cannot be expected to give absolutely exact values, but rightly handled, it gives as exact values as it is possible to reach under any circumstances.

It is easy to see the significance of a reduction in accordance with this formula. The number of cells with a mean transverse diameter of 1 mm. on v. Economo's photomicrographs (magnification 100:1 and section depth of 25μ) is thus to be multi-

plied by 0.8, those with a diameter of 2 mm. by 0.6 those with a transverse diameter of 5 mm. by 0.333 etc.

The formula has general validity and can be used for different bodies and formations with rounded shapes such as granula, cell nuclei, Langerhans' islands, and such. On its own showing, however, the results will be more exact, the nearer the counted bodies approach or are reduced to the spherical form. It is therefore decidedly more advantageous to count and measure the nerve cell nuclei than to take the actual nerve cells in the cortex.

The results from the calculations suggested above of the number of nerve cells in transverse sections of the cortex are arranged, as already described, by systematic tabulation, whereby the number of equal-diameter nerve cell nuclei in each squared field are entered in corresponding columns. The different squared fields had been arranged and numbered as in the tables on photomicrographs (magnification 100:1). This arrangement makes it possible to determine the number of nerve cells in a volume unit from the part of the cortex desired. The counted squared fields from this part are assembled, the number of nerve cells with the same diameter are added up, and the totals are reduced with the help of the formula described above. The number of nerve cells obtained by the addition of the reduced totals is to the nerve cell number of the volume unit under consideration as the section volume corresponding to the squared fields included is to the volume of the unit in question. The unknown quantity (x), the volume unit's nerve cell figure, is obtained from the three other known factors making up the equation. It is in the nature of the case that the value we are looking for, (x), becomes surer, the surer the other three factors are.

The reader has no doubt realized that it is a laborious and very complicated task to make a calculation of the total number of nerve cells in the cortex, whether it concerns a volume unit of the thickness of the whole cortex, some one of its laminae or the whole cortical region of the brain. If, however, the investigation is worth carrying out at all, it is

worth carrying out with methods which can lead to correct results, and this, according to what has been said above, is not possible with the method followed by v. Economo and Koskinas.

In the problem raised by Lindgren ('40), which amounts to a comparison between the cyto- and angio-architectonics of the cerebral cortex, one ought of course to carry out quantitative capillary and nerve cell determinations on sections which have been stained for nerve cell and which come from parts of the cortex in which the capillaries have been completely injected.

SUMMARY

In connection with an expert examination, the author has had to define his position to the figures of v. Economo and Koskinas for the number of nerve cells per volume unit in the human cortex. The author shows that these nerve cell figures are impaired by such methodological deficiencies that they cannot be accepted even as approximately accurate. A method of proceeding is sketched for numerical determinations of this kind, which, rightly handled, leads to results as exact as it is possible to reach. In this connection a reduction formula is submitted, which can be considered very usable in numerical and quantitative micromorphological calculations.

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A MEDIAN VAGINAL CANAL AND OTHER ANOMALIES OF THE GENITAL TRACT OF THE OPOSSUM, *DIDELPHYS VIRGINIANA*

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FOUR FIGURES¹

The genito-urinary organs of various species of Marsupials have been described from time to time during the past two centuries (Lister and Fletcher, 1881; Hill, 1899, '00; Hill and Fraser, '25) with especial attention to generic and specific variations upon the anatomic pattern common to the group, as well as to changes that occur with maturation and breeding. Apparently, however, the possibility of variations in the structure of the genital tract of females of any one species was ignored, and perhaps rightly so, for there was no obvious reason why Marsupials should differ in this respect from other mammals. At least, I assumed this to be true at the outset of a series of autopsies of opossums, *Didelphys virginiana*, but after a clearly marked median vaginal canal was found in an animal that had been reared in captivity and never borne young, the genital tract of all females was more completely explored.

These examinations have shown that a median vaginal canal is not particularly rare in *D. virginiana* and that other, less conspicuous, anomalies occur with some regularity. In view of the embryonic origin of the genital tract of the Virginia opossum (Baxter, '35) possibly these developmental irregularities are not entirely unexpected, but, because of the route believed to be taken by offspring of this and other species of

Marsupials in leaving the uteri (Hill, 1899, '00; Hartman, '20) they may be of some interest.

The anomalies that are to be described were found in a series of fifty-two animals that died, or were killed, in the course of other studies. Relatively few specimens were suitable for histologic examination because of post mortem changes.

The usual arrangement of the genito-urinary canals of *D. virginiana* are shown in figure 1, A, as they appear from the ventral surface. In this outline sketch the bladder (bl.) has been displaced to the right and minor details omitted. From

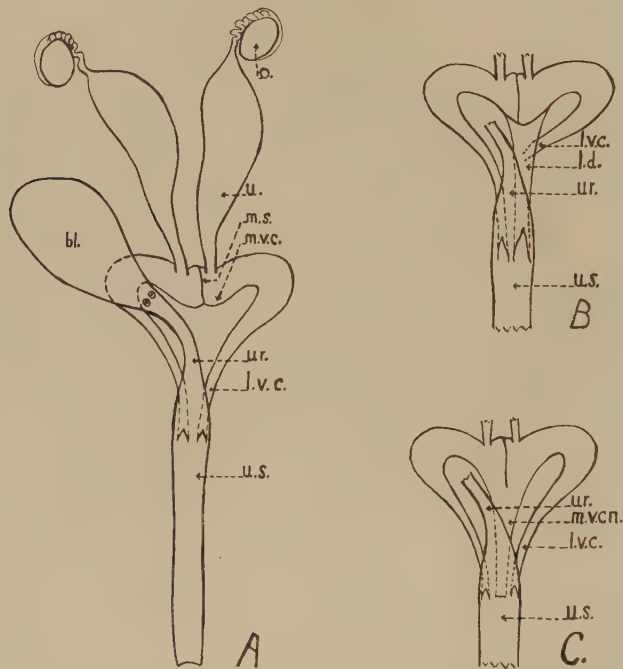


Fig. 1 A, Diagram of female genital organs of *Didelphys virginiana* from the ventral surface. u.s., urogenital sinus; ur., urethra; l.v.c., lateral vaginal canals; m.v.c., median vaginal culs-de-sac; u., uterus; bl., bladder; o., ovary. B, Diagram from the ventral surface of the mid-portion of the genito-urinary canals of a female opossum, *D. virginiana* in which a lateral diverticulum—l.d., of the urogenital sinus had developed. C, Diagram from the ventral surface of the mid-portion of the genito-urinary canals of a female opossum, *D. virginiana*, in which the median vaginal canal—m.v.cn., was present.

its external opening the urogenital sinus (u.s.) runs cephalad for 5 or 6 cm., then divides into the urethra (ur.) and lateral vaginal canals (l.v.c.). The urethra is from one-third to one-fourth the length of the urogenital sinus, and not the short structure represented in the drawing by McCrady ('38), joining the lateral vaginal canals immediately below the neck of the bladder. The openings of the lateral vaginal canal vary from lateral to dorso-lateral positions. Often they are not approximately opposite and in one animal the distal part of the right canal crossed ventral to its fellow, entering the urogenital sinus through the left ventral face of the tube.

The openings of the lateral vaginal canals also may vary in size. Usually they are small, simple v-shaped spaces in the mucosa of the urogenital sinus to which the wall of the lateral canals joins without distinct interruption. In two animals these openings were much larger, approximately equaling the diameter of the urethra and forming pockets into which the ends of the lateral canals projected. In a third animal (fig. 1, B) the right lateral vaginal canal also opened into a wide pocket, but on the left side a cephalad extension of the urogenital sinus replaced the ostium of the lateral canal and extended to the membranous wall of the median vaginal culs-de-sac (m.v.c.) as a voluminous, thin-walled diverticulum (l.d.). The lateral vaginal canal was distinctly shortened on this side and opened into this diverticulum through the dorsal wall, about 1 cm. above the level at which the other vaginal canal joined the urethra.

Short median diverticula, or cephalad extensions of the urogenital sinus, occurred in four other animals. These arose in the dorsal mid-line of the tube at about the level of the openings of the lateral vaginal canals, and measured 10 to 15 mm. in length, by 3 to 4 mm. in diameter. They probably would have been overlooked had they not interfered with the passage of a catheter into the bladder.

The median vaginal canal (m.v.cn., fig. 1, C) was found in five animals of this series, as a delicate, thin-walled tube which also entered the urogenital sinus at about the level of

the lateral vaginal canals (l.v.c.). An inconspicuous transverse slit, 2 to 3 mm. in width, on the dorsal wall of the tube marked the junction of the two passages. From this point the median canal passed dorsal to the urethra and joined the median vaginal culs-de-sac (m.v.c.) to form a single chamber in which the median septum (m.s.) was incomplete.

In sections the median tube appeared as an inconspicuous space in an area of loosely arranged, cellular connective tissue lying between the lateral vaginal canals (fig. 2). Its lining cells were small, closely placed columnar epithelium which formed a delicate, single layer (fig. 3). Immediately adjacent to this median canal were occasional gland-like spaces also lined by small columnar epithelial cells. By way of contract figure 4 is from a section taken at about the same level in the genital apparatus of an animal which lacked the median canal.

During the examinations of this series of animals faults in the development of the median septum between the median vaginal culs-de-sac were noted. This structure was completely absent, incomplete at some peripheral point, or perforated by one or more oval openings. Records do not permit an estimate of the frequency with which these various defects occur.

With one exception variations in the size and position of the openings of the lateral vaginal canals into the urinary passage probably are within the limits of normal development. But, median extensions of the urogenital sinus, as well as the development of median vaginal canals, seem to be anomalies of another order.

The promixal parts of the lateral vaginal canals are formed from the müllerian ducts which, as they approach the urogenital sinus, stimulate growth from the region of the old cloacal horns to form the distal third of canals (Baxter, '35). Perhaps a median vaginal canal might develop by caudad extension of the median parts of the lateral vaginal canals which form the median culs-de-sac so that contact is made with the wall of the urogenital sinus. If this be true, then

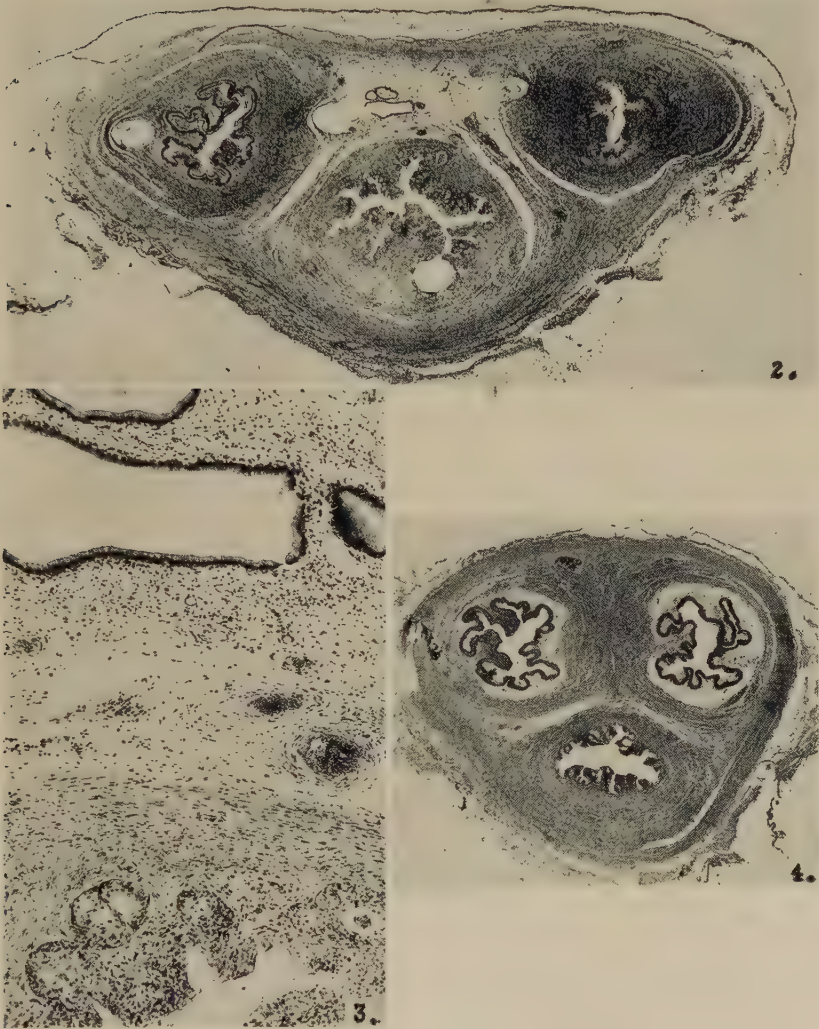


Fig. 2 Transverse section of genital cord of a female *Didelphys virginiana* showing urethra, lateral and median vaginal canals. Circular openings visible in the tissue mark the path of a probe. This section was taken at the level marked ur. in figure 1 A. $\times 7$.

Fig. 3 An enlarged view of part of the median vaginal canal showing lining epithelium, surrounding glands and stroma and part of adjacent urethra. $\times 80$.

Fig. 4 Transverse section of genital cord of a female in which the median vaginal canal did not occur. $\times 7$. (Reduced by approx. 12%.)

median diverticula of the urogenital sinus would represent incomplete union of the two parts of this anomalous channel.

Possibly the partial or complete development of the median vaginal canal in *Didelphys virginiana* should not be regarded as entirely abnormal, but simply an occasional reappearance of a feature common to ancestral forms, and still occurring in some contemporary Marsupials. This passage develops regularly in species of *Macropodidae* (Fletcher, 1881; Lister and Fletcher, 1881). Likewise, intercommunications between the median vaginal culs-de-sac occur in other Australian Marsupials, *Perameles* and *Dasyurus*, which lack the median vaginal canal (Hill, 1899; '00) and in two species of *Didelphyidae*, *Monodelphis americana* and *Marmosa cinerea* (Hill and Fraser, '25).

The finding of a median vaginal canal in *D. virginiana* is contrary to other observations (Hartman, '20; Hill and Fraser, '25). Possibly these workers did not examine a sufficiently large series of animals. The fact that this passage occurs in some 10% of the present series leads one to question the description of parturition in this species (Hartman, '20). It is said that, in leaving the uteri, the embryos avoid the lateral vaginal canals and break through the median vaginal culs-de-sac, force a passage through the intervening tissue to the wall of the urogenital sinus, then break through this wall to gain the exterior. The young of *Perameles* (Hill, 1899) and *Dasyurus* (Hill, '00) also are believed to take this route at birth.

Without more evidence than the occurrence of a median vaginal canal in occasional animals one hesitates to question these observations, but, if the normal exit from the uteri be by way of a "pseudovaginal canal" (Hill, 1899), one wonders that a negatively geotropic foetus with only the fore limbs developed for locomotion can force this passage, especially so, when full-term embryos, still enclosed by foetal membranes may be aborted (Hartman, '20). Certainly these must have followed preformed channels.

SUMMARY

In five of a series of fifty-two female opossums, *Didelphys virginiana*, a median vaginal canal formed a direct communication between the vaginal culs-de-sac and the urogenital sinus. Two of these animals had never borne young. This tube passed dorsal to the urethra in the median line and opened into the urinary passage at about the same level as the lateral vaginal canals. It was lined by columnar epithelium and lacked a muscular coat. Other variations from the usual structure were median dorsal extensions of the urogenital sinus (four cases) and displacement of one lateral vaginal canal (one case).

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CHANGES IN THE LIPOID CONTENT OF THE ADRENAL GLAND OF THE RAT UNDER CONDITIONS OF ACTIVITY AND REST

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ONE PLATE (SEVEN FIGURES)

INTRODUCTION

It has been noted previously (Selye, '36 a and b, '37 b) that during the general adaptation syndrome, the adrenal glands show quite marked and characteristic changes. Hypertrophy of the cortex and loss of lipoid occurs during the stage of "alarm." This is followed by a return to a relatively normal condition with an increase in lipoid content during the stage of resistance and later by a continued hypertrophy with a second decrease in lipoid content at the end or exhaustion stage. These changes have been noted regularly and are quite obvious in animals brought to autopsy. Adrenals from animals in the stage of "alarm" and exhaustion are brown and considerably enlarged while those from animals in the resistant stage have a pale yellow color resulting from the increased lipoid content. These observations, supported by evidence from the study of frozen sections of this material stained with Sudan III, lead to the conclusion that hypertrophy and loss of lipoid was associated with increased activity, an increase in lipoid with rest or inactivity. Thus, the amount of lipoid present in the adrenal cortex would vary greatly during the different stages of the adaptation syndrome.

In this journal Flexner and Grollman ('39), apparently unaware of the above mentioned investigations, concluded from a study in which the degree of reduction of osmic was

used to determine the relative amounts of lipid in the adrenal cortex, that "In general, depression of activity is correlated with a decrease in the reduction of osmic acid; stimulation of activity with an increase in the reduction of osmic acid." It seemed advisable therefore, to repeat and extend earlier experiments, using both osmic acid and Sudan III to determine the relative lipid content of the adrenal cortex under conditions known to induce activity or rest.

MATERIAL AND METHODS

Male white rats averaging 125-150 gm. were used throughout the experiments. They were fed on a standard purina diet. In all cases the left adrenal was preserved in 4% formaldehyde and weighed. Frozen sections were then made from this adrenal and stained with Sudan III. The right adrenal was in most cases fixed and post-osmicated following the method used by Flexner and Grollman ('39).

RESULTS

Eleven groups of six rats each were studied and were as follows: (1) normal fed rats; (2) rats fasted 24 hours then killed; (3) fed, killed 1 hour after the subcutaneous injection of 0.4 cc. of 4% formaldehyde; (4) fasted for 24 hours, treated with 0.2 cc. of 4% formaldehyde at the twentieth and twenty-third hours and killed on the twenty-fourth hour of the fast; (5) fasted 24 hours, treated with 0.2 cc. of 4% formaldehyde at the sixth, twelfth, eighteenth and twenty-third hours of the fast and killed on the twenty-fourth; (6) treated with three subcutaneous injections of 0.2 cc. of 4% formaldehyde daily for 7 days and fasted during the last 24 hours; (7) fed, killed after exposure to cold (-3° to -5°C.) for 1 hour; (8) fasted for 24 hours, exposed to cold from the twentieth to the twenty-fourth hour of the fast; (9) fasted and treated with cold for 24 hours; (10) treated with cold for 7 days, fasted during the last 24 hours; (11) treated with cold for 13 days and fed throughout.

TABLE 1

GROUP	TREATMENT	LIPOID CONTENT AVERAGE 6 RATS	ADRENAL CONTENT AVERAGE 6 RATS
1	Control fed	++++	36
2	Untreated fast 24 hours	+++	38
3	Form. 1 hour fed	++	33
5	Form. 24 hours fasted	++	42
6	Form. 7 days (fasted)	++++	52
7	Cold 1 hour fed	++	32
9	Cold 24 hours fasted	++	44
10	Cold 7 days (fasted)	+	50
11 ¹	Cold 13 days fed	+++	32

¹ Average body weight 125 gm. All other groups average weight of 150 gm.

A comparison of the Sudan III and osmic acid preparations indicates that while osmic acid failed to impregnate certain of the lipoids stainable with Sudan III, the general results with the two methods are in complete agreement.

The results summarized in table 1 clearly demonstrate several points. A 24-hour fast definitely reduces the lipid content of the adrenal cortex (compare figs. 1 and 2). Treatment with formaldehyde or cold for 1 hour causes a much greater loss of lipid (compare figs. 1, 2 and 3). (Though not indicated in the table, treatment for the longer period of 4 hours results in a temporary increase in lipid—figs. 3 and 4.) Continued treatment for 24 hours results in a decrease in lipid content to well below normal levels (figs. 2 and 5). Treatment with formaldehyde over a period of 7 days results in a return of the lipid content to normal or above normal levels (figs. 2 and 6). Similar results are obtained by treatment with cold over a period of 13 days providing the animals are fed throughout. However, treatment with excessive cold over a period of 7 days with fasting during the last 24 hours results in a condition very like exhaustion with loss of lipid (figs. 2 and 7). A further result not indicated in the table has been the confirmation of the observation that there is a great increase in the lipid content of the cells of the adrenal cortex for a short period after hypophysectomy (Selye, Collip and Thompson, '35). A comparison of the average weights of the adrenals in the various groups shows

quite clearly that hypertrophy of the gland occurred in all cases where treatment was continued for any length of time. It may be noted even after 4 hours treatment.

DISCUSSION

The results reported above give further support to the interpretation of the experiments mentioned previously (*loc. cit.*). Nonspecific damage (in the present experiments, treatment with cold and formaldehyde) at first stimulates adrenal cortical activity as evidenced by hypertrophy and loss of lipoids. The organism eventually becomes resistant to continued treatment and when this occurs, cortical hormone is no longer needed in excess. As a result, lipoids are again stored in the relatively inactive cortex. Finally after still further treatment, this resistant stage ends and a stage of exhaustion sets in, at which time excessive amounts of cortical hormone are again required. As a result, lipoids again decrease and hypertrophy continues. Thus we interpret hypertrophy and decrease in cortical lipoid as evidence of increased cortical activity and increase in cortical lipoid as evidence of a condition of relative inactivity and rest.

The observation that more lipoid appears to be present after 4 hours treatment than after 1 hour or 24 hours is of interest. Our results in this instance corroborate the findings of Flexner and Grollman ('39). It is possible that due to the relatively light treatment, the animals in the 4-hour groups were already entering the resistant stage when sacrificed, this tendency being reversed with continued treatment. Whatever the explanation, this observation does not affect the general conclusion that cortical activity is associated with hypertrophy and decrease in lipoid content.

These results and our interpretation of them are for the most part, not in accord with those of Flexner and Grollman ('39) who, as stated previously, concluded from a somewhat similar investigation that increased cortical lipoid indicates activity while decreased lipoid content indicates decreased activity and exhaustion of the adrenal cortex. It is rather difficult for us to understand how decreased activity and ex-

haustion could possibly exhibit similar cytological characteristics since exhaustion as such might be expected to result from long continued over-activity.

The results of Miller and Riddle ('39), are in complete agreement with our own. Working with the pigeon, they found a relative increase in the lipid content of the cortical cells after hypophysectomy, together with involution of the cortex and degenerative changes in the mitochondria and Golgi apparatus of the cells. They found further, that after hypophysectomy stimulation of the cortex by means of adreno-tropic factor of the pituitary resulted in a reversal of the above changes, i.e., hypertrophy and loss of cortical lipoids and regeneration of mitochondria and the Golgi apparatus.

Further, it must be considered of significance that hypertrophy and loss of lipoids in the rat occurs at the time when the need for cortical hormone has been shown to be greatest (Selye, '37 a and c). If further evidence be needed, Weil and Browne ('39), using the bioassay method of Selye and Schenker ('39), found greatly increased cortin excretion after major surgical interventions. This occurs at a time comparable to the cortical hypertrophy in experimental animals. Our interpretation receives further support from what is known concerning the activity of the corpus luteum. During the functional life of this gland progesterone production is at its height at the time when the amounts of stainable lipoids are at a minimum.

SUMMARY

1. Stimulation of the adrenal cortex by treatment with cold and formaldehyde results in hypertrophy of the cortex and decrease in its lipid content.

2. During the resistant stage of the adaptation syndrome when cortical activity is decreased, lipid is again stored.

3. In exhaustion, the cortical hormone is again needed in excessive amounts and as a result, cortical hypertrophy continues with an associated loss of lipoids.

4. It is our opinion that most of the evidence supports the contention that hypertrophy of the adrenal cortex with a

decrease in its lipoid content is always related to increased activity and increased production of the adrenal cortical hormones.

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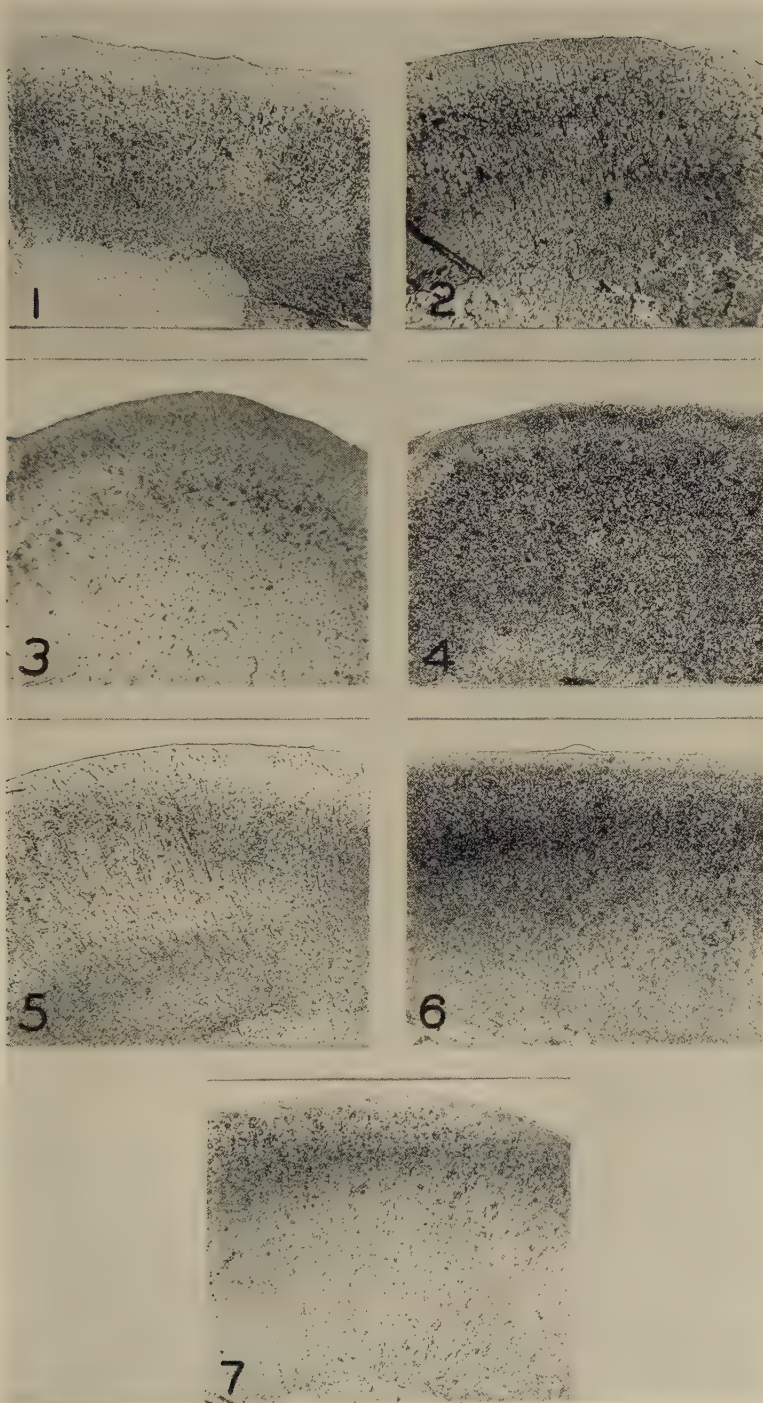
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PLATE 1

EXPLANATION OF FIGURES

All figures photomicrographs of rat right adrenal glands fixed and post-osmicated after the method of Flexner and Grollman and sectioned at 8μ . Magnification approximately $\times 40$.

- 1 Adrenal of normal fed rat. Relatively large amounts of osmiophilic material (lipoid) in the fasciculata and reticularis of the cortex are apparent.
- 2 Adrenal of an untreated rat fasted 24 hours. There is a definite reduction in the amount of lipoid material. The large dark masses between the cortical cells in this case are red blood corpuscles which have reduced the osmic acid.
- 3 Adrenal from a fed rat after 1 hour's treatment with formaldehyde. Note the decrease in lipoid.
- 4 Adrenal from a rat fasted 24 hours treated with formaldehyde for 4 hours. An increase in the lipoid material is evident.
- 5 Adrenal from a rat fasted and exposed to cold for 24 hours. Note the decrease in cortical lipoid.
- 6 Adrenal from a rat brought to the stage of resistance by continued treatment over a period of 7 days with formaldehyde. The lipoid has again increased to normal or above normal levels.
- 7 Adrenal from a rat in a condition simulating exhaustion due to continued treatment with cold over a period of 7 days and fasted during the last 24 hours. A decrease in lipoid is evident. Animals not fasted at the end of such treatment show the normal amount of lipoid.



PROTEOLYTIC ENZYME ACTIVITY OF COLLOID EXTRACTED FROM SINGLE FOLLICLES OF THE RAT THYROID ¹

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ONE PLATE (FIVE FIGURES)

Knowledge of the properties and composition of thyroid colloid is fragmentary. It was thought that studies of colloid extracted from single follicles of the thyroid gland would help to fill this gap. Experiments of this sort were performed with microdissection techniques on the thyroid gland of living rats. As a result it becomes possible to reinterpret the appearance of the colloid in fixed preparations in the light of its state in the living animal. In addition, the results support earlier evidence that colloid may be reabsorbed from the follicles by means of the previous action of a proteolytic enzyme present in this fluid. This enzyme is thus a second known component of colloid, the other being thyroglobulin.

According to this concept, thyroglobulin is hydrolyzed in the lumen of the follicle and the smaller molecules resulting from this hydrolysis become available for transport through the follicular cells. It was determined that there are fluctuations in the extent of proteolytic activity not only in different follicles of normal glands but also (to a greater extent) under certain experimental conditions: i.e., after the administration of potassium iodide and of thyrotropic pituitary

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² Fellow of the Rockefeller Foundation.

extract. Estimates of the pH of the colloid fluid served to show that such changes in enzymatic activity seem to be independent of this factor.

METHOD

Rats were anesthetized with nembutal, supplemented occasionally with ether. The thyroid gland was exposed, and the isthmus illuminated from above. In this portion of the gland the follicles are disposed in few layers deep, so that micromanipulation is simplified. Colloid was aspirated sometimes from lobar follicles. Micropipettes with a bore of 5–20 μ were used. Contamination by tissue fluid was prevented by slight drying of the surface (as recommended by Wearn and Richards, '24-'25) and by filling the pipette to the tip with mercury.

The aspirated colloid was diluted in the micropipette with five to ten times its volume of buffer and tested for the presence of proteolytic activity by the method recommended by Pickford and Dorris ('34). Lantern slide plates are cleared of silver salts, washed in running water, dried and cut into thin strips. The buffered colloid, which forms a droplet of the order of 0.5 mm. in diameter, is carefully deposited on the gelatin film under the binocular microscope. The plate is transferred rapidly to a moist chamber where it is kept at 24–25°C. for 2 hours. After this time the slides are rinsed with distilled water followed by washing in running water for several hours. This procedure stops digestion and effects a uniform swelling of the gelatin film. The slides while wet were stained with 1% acid fuchsin for 5 minutes, rinsed in distilled water and 95% alcohol, and then dried. Observations of the degree of digestion were made with low magnifications of the microscope. The degree of digestion was judged by the depth of staining in the crater formed by the sample droplet. Control digestions with buffer alone yielded negative results. Confirming Pickford and Dorris, it was found that Pancreatin Merck produces strongly positive digestion in a dilution of 1:1000; the reaction is positive in a dilution of 1:5000.

As these investigators pointed out, this micromethod is not quantitative. In addition, it is very difficult to aspirate exactly the same amount of colloid to be diluted with the same volume of buffer solution from different follicles. Nevertheless, we believe that it is possible to make a semiquantitative estimation of changes in enzymatic activity. The results were estimated as negative (—), faintly positive ($\pm$), positive (+), strongly positive (++), and total digestion (+++).

A relative estimate of the viscosity of colloid was made on the basis of the ease or difficulty in aspirating and expelling the colloid from the pipette.

In order to determine the pH of the colloid, indicators of the Clark and Lubs series were injected into the follicle with a small-bore pipette. Injections were also made in the perifollicular connective tissue. In some cases the time of diffusion of the indicator within the follicle, and of its subsequent disappearance, was measured. The indicators were made up in saturated aqueous solution. This seemed a justifiable way of increasing color density because the size of the droplet injected was so minute as compared with the volume of colloid in the follicle.

OBSERVATIONS

Colloid and enzyme activity in normal rats. The colloid aspirated from follicles of normal rats is very viscous: that is, it rises in the micropipette very slowly and with a high negative pressure. It is quite brilliant and always unquestionably fluid. Sometimes the droplets appear faintly yellow with reflected light, but generally they are colorless. The colloid is optically homogeneous and its viscosity in different parts of single follicles seems to be uniform. No vacuoles were observed at any time in the colloid even on observation with high powers of the microscope. Contamination with blood cells from the perifollicular capillaries was observed in very few cases; in these, the erythrocytes were easily visible with the binocular microscope. Vascular injury seems to be minimized by closure of the perifollicular capillaries as a result of compression by the pipette.

The proteolytic activity of colloid from different individual follicles of normal rat glands is shown in table 1. It was tested in the "physiological range" of pH, between 7.4 and 6.6. In all cases the reaction was positive but not very marked. These results were checked with the buffer solution alone and by denaturing the protein with formalin and mercuric chloride. In these three latter procedures the results were

TABLE 1
Proteolytic activity of colloid in normal and experimental animals

RAT NO.	WEIGHT IN GRAMS	ACTIVITY OF ENZYME		TIME AFTER ADMINISTRATION OF KI OR THYROTROPIC EXTRACT	DOSAGE OF THYROTROPIC EXTRACT IN G.P. UNITS
		pH of buffer 7	pH of buffer 6.6		
Normal rats					
1	115	+, +			
2	150	+, +			
3	155	+, +			
4		±, +, +			
5	120		+		
6	105		+		
Rats treated with potassium iodide					
7		+	++	5 days	
8	93	+++	++	7 days	
9	70	+	+, ++	10 days	
10	90	++, ++		10 days	
11	90	++		11 days	
12	100	++		12 days	
13	83	+, ±		45 days	
14	93	—, ±		46 days	
15		±		50 days	
16	93	±, ±		54 days	
Rats treated with thyrotropic pituitary extract					
17	105	++, +, +	++, ++	5 hours	10
18	100	++, ++	+, ++	6 hours	13
19	107	++, ++	+	10 hours	10
20	125	++		16 hours	10
21	107	+++, +, +	+	20 hours	20
22	80	++, ++		24 hours	10
23	105	+, ++, ++		28 hours	15
24	125	+, +	±, ±	44 hours	15
25	93	+, +, +		48 hours	20

— negative, ± faintly positive, + positive, ++ strongly positive, +++ total digestion.

negative. In rat 4 of table 1, colloid was permitted to act on the gelatin film for $\frac{1}{2}$, 1 and 2 hours.

Action of potassium iodide on the activity of the proteolytic enzyme. Rats fed a normal diet with 1% of potassium iodide added to the ground food showed a marked change in the properties of the colloid and in the activity of the proteolytic enzyme (see table 1). The results differ according to the time during which potassium iodide was administered. One group of six rats was so fed for a period of 5 to 12 days; the second group of four rats was treated for 45 to 54 days. In the first group, vascularity was improved, the colloid was less viscous, and the activity of the enzyme was greatly increased (table 1). In the second group the colloid was highly viscous; in fact, it seemed sometimes more viscous than in normal glands. Coincidentally, the activity of the enzyme was decreased below the normal level (table 1).

Activity of the enzyme after injection of pituitary extract containing thyrotropic factor. Rats were injected intraperitoneally with doses of thyrotropic pituitary extract ranging between 1 and 2 mg. of dry weight (1 mg. contained 10 guinea pig units) in one or two injections. Animals were studied between 5 and 48 hours after the first injection.

The follicles in all cases showed increased vascularity with marked dilatation of the perifollicular capillaries. The viscosity of the colloid was greatly decreased: it was very easily aspirated in the micropipette. The refractive index seemed to be diminished. In general, the activity of the proteolytic enzyme was increased especially when the colloid was taken a short time after the injection (5-24 hours) (table 1). In these cases, the results generally were strongly positive.

Nevertheless, in two cases when colloid was aspirated a longer time after the administration of the pituitary extract (44-48 hours), the activity fell within the limits of the normal animal. No record of the viscosity of the fluid were made in these two rats.

Effect of the pH of the buffer on the activity of the enzyme. Several scattered observations are summarized in table 2. The

general trend is for the activity of the enzyme to increase greatly in the acid range, especially at pH 3 and pH 2. At pH 8.2, the activity seems to be lower than within the physiological range. This variation of enzymatic activity with pH is seen in the colloid of normal and also experimental rats.

TABLE 2
The effect of changes in pH on the proteolytic activity of colloid

RAT NO.	ACTIVITY OF ENZYME					TIME AFTER ADMINISTRATION OF KI OR THYROTROPIC EXTRACT	DOSAGE OF THYROTROPIC EXTRACT IN G.P. UNITS
	Buffer pH 2	Buffer pH 3	Buffer 4-5	Buffer 6-7.4	Buffer 8.2		
Normal rats							
6a	++	+++	+	+	+		
26	++				—		
27	++				+		
28	++						
29	++, ++						
Rats treated with KI							
7				++, +	+	5 days	
8				++, +++	++	7 days	
13	+			+, ±		45 days	
14	+			±, —		46 days	
15	+	+	+	±, —		50 days	
Rats treated with thyrotropic pituitary extract							
17				++, ++			
				++, +, +	+	5 hours	10
18		++	++	++, ++			
				++, ++	+	6 hours	13
20			++	+, ++, ++		16 hours	10
25	++			+, +, +		48 hours	20

— negative, ± faintly positive, + positive, ++ strongly positive, +++ total digestion.

The pH of the intrafollicular colloid and of the perifollicular tissue. Table 3 gives a general idea of the results found in follicles of more than twenty normal and experimental rats. Four indicators with overlapping pK were injected: (chlor-phenol red, brom-cresol purple, brom-thymol blue, and phenol red).

Brom-thymol blue gave the most striking results. When injected into the follicle its color is yellow-green or green

(pH 6.5–6.7) and when injected outside of the follicle the color is blue (pH 7.1). The other indicators checked with these results in all cases. The use of electrometric methods for the determination of the pH of colloid would give more accurate results. Nevertheless, the fact that the indicators used overlap in their effective ranges permit of some confidence in the determinations reported in this paper.

While the indicator is injected into the colloid it spreads through the whole follicle and reaches the periphery in 30 to 90 seconds, after which it appears optically uniform in density. Thereafter, the color intensity decreases regularly and uniformly over the whole follicle and after 3 minutes 30

TABLE 3

pH of the intrafollicular colloid and of the perifollicular connective tissue

	CPR		BCP		BTB		PR		FINAL pH
	Color	pH	Color	pH	Color	pH	Color	pH	
Intra-follicular colloid	Red	>6.3	light purple	6.6	yellow green	6.5–6.7	yellow	<7.0	6.6 ± 0.2
Peri-follicular tissue	Red	>6.3	dark purple	>7.0	blue	7.3–7.5	pink	7.4	7.3 ± 0.2

CPR, chlor-phenol red; BCP, brom-cresol purple; BTB, brom-thymol blue; PR, phenol red.

seconds to 15 minutes the indicator disappears from the follicle. This is probably due to the diffusion of the indicator through the follicular wall. When it is injected just before the death of the animal it remains unchanged in the follicle for at least 30 minutes. The indicator injected in the perifollicular connective tissue of the living gland with good circulation also diffuses, and disappears very rapidly, in about 1 or 2 minutes.

DISCUSSION AND SUMMARY

The application of microdissection methods to the thyroid gland of the rat has resulted in more direct information concerning the colloid. Colloid appears in the follicles of normal

animals as a highly viscous but fluid substance with high refractility. The fact that the indicator injected in the follicles spreads rapidly from the point of injection throughout the whole follicle, also is a confirmation of the fluidity of the colloid. It seems to be completely homogeneous, with no differences in viscosity within a single follicle. There is no evidence for the existence of vacuoles in the colloid. These observations confirm the results obtained by Gersh and Caspersson ('40) and by De Robertis ('41) who used the freezing-drying method to prepare their sections. In addition observations "in vivo" of the thyroid gland by Vonwiller and Wigodskaya ('34), Hartoch ('33), Bucher ('38) and Williams ('37) had previously emphasized the general optical homogeneity of colloid. Thus evidence from these several sources makes it appear quite clear that the peripheral colloid vacuoles described in most preparations made with usual methods, are artifacts. This conclusion is contrary to the implications of many histologists working with customary fixatives and stains, that colloid is of a "solid" nature.

Under certain experimental conditions the viscosity of the colloid is changed. After administration of potassium iodide for a few days the viscosity clearly is below the normal, confirming the indirect observations of Loeb et al. ('29). The viscosity increases however, after prolonged treatment with the potassium iodide, confirming also the observations of Loeb ('29) of a secondary increase in the density of the colloid. The administration of thyrotropic pituitary extract produces a sharp decrease in the colloid viscosity which is noticed clearly as soon as 5 hours after the injection.

Changes in the viscosity of the colloid are correlated with alterations in its proteolytic activity. These observations are a direct demonstration of the assumption of Loeb ('29) when he stated that "the softening and solution processes (in KI administration) are caused by a substance, presumably of an enzymatic character which is given off by the epithelial cells when they are in a state of stimulation." He assumed also

that secondarily this enzymatic substance is not secreted and the colloid becomes solid.

The work of Lerman and Salter ('34) was the first that suggested the possibility that colloid might be reabsorbed from the follicle after enzymatic hydrolysis. They report some experiments in which extracts of thyroid tissue were used to synthesize thyroid hormone *in vitro* and they suggested the possible existence of an enzymatic mechanism which could catalyze either protein formation or protein destruction according to thermodynamic conditions. It is necessary to emphasize that the results of Lerman and Salter were obtained with extracts of the whole gland while in this paper enzymatic activity of isolated colloid is reported.

The same mechanism was invoked by Gersh and Caspersson ('40) as the only one capable of accounting for the great variability of colloid protein and organic iodine in normal and experimental animals. It was difficult otherwise to understand how a molecule with such a large molecular weight as thyroglobulin could pass with such rapidity through the cells lining the follicle. The existence of this mechanism now is greatly strengthened by the observations reported in this paper.

The presence of such proteolytic enzyme activity in the colloid was clearly established. The fluctuations in the amount of enzymatic activity, depending on experimental conditions, support the concept that in the reabsorption of colloid a proteolytic enzyme is secreted by the thyroid cells which hydrolyzes the large protein molecule of thyroglobulin. The reference to one proteolytic enzyme is a convenience; the existence of more than one enzyme is not excluded by the methods employed. Results cited by Lerman and Salter indicate that the enzyme or enzyme system is probably non-specific since proteases have been detected in extracts from other organs. The results obtained by the injection of indicators permit us to affirm that these fluctuations in the activity of the proteolytic enzyme in the experimental animals are probably not due to changes in the pH of the colloid. Theoreti-

cally, it seems possible that the proteolytic enzyme is also able to synthesize the thyroglobulin and that a balanced equilibrium may exist in the colloid between those two reactions. In the colloid of follicles activated by the pituitary extract this equilibrium is shifted further toward the hydrolysis side and the total protein concentration diminishes. The latter is established by the measurements of Gersh and Caspersson for the guinea pig. In the colloid of follicles of rats treated with potassium iodide, the equilibrium is first in the same direction (primary activation); later, the direction is shifted in the opposite way (secondary period). The latter stage of increased protein content in the colloid has been described by Gersh and Caspersson for the guinea pig. It seems also possible that a similar enzymatic mechanism may exist in the secretory cells of the thyroid gland. A better knowledge of the factors that regulate the equilibrium of these enzymatic reactions in colloid and cells may throw light on the secretory mechanism of the thyroid gland in normal, experimental, and pathological states.

CONCLUSION

1. By microdissection methods colloid was extracted from single follicles.

2. The colloid is an optically homogeneous, highly viscous, refractile fluid. Its viscosity diminishes after the injection of thyrotropic pituitary extract and after a short period of feeding with potassium iodide. After a longer period of iodide administration the viscosity increases.

3. The pH of the colloid, determined by the microinjection of indicators, is 6.6 ± 0.2 ; that in the perifollicular tissue: 7.3 ± 0.2 . No marked changes in pH were found after the administration of potassium iodide or thyrotropic pituitary extract.

4. Diffusion of the indicator in the follicle takes place in 30 to 90 seconds; it is reabsorbed in 3 to 15 minutes.

5. In the colloid obtained from single follicles is present a proteolytic enzyme which digests a gelatin substrate.

6. This proteolytic enzyme increases in activity in the acid range and diminishes in the alkaline. Within the physiological pH range, the activity increases after injection of thyrotropic pituitary extract and after the administration of potassium iodide for a short time. After treatment with iodide for longer time, the activity decreases below the normal level.

7. The demonstration of this proteolytic enzyme, and the fluctuations in its activity in experimental conditions, support the concept that an enzymatic mechanism is involved in the hydrolysis of the colloid protein and the subsequent reabsorption of the products of hydrolysis.

I wish to acknowledge my indebtedness to Dr. I. Gersh for the suggestion of the problem and for his advice and criticism, to Dr. H. Jensen of the Squibb Institute for Medical Research for the thyrotropic pituitary extract, and to this laboratory for the facilities and courtesies I have received.

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PLATE 1

EXPLANATION OF FIGURES

1 Proteolytic activity faintly positive ($\pm$) of colloid from a rat fed with potassium iodide during 45 days (pH 7). The clear ring and the central clear spot observed in this picture are due to folding of the gelatine.

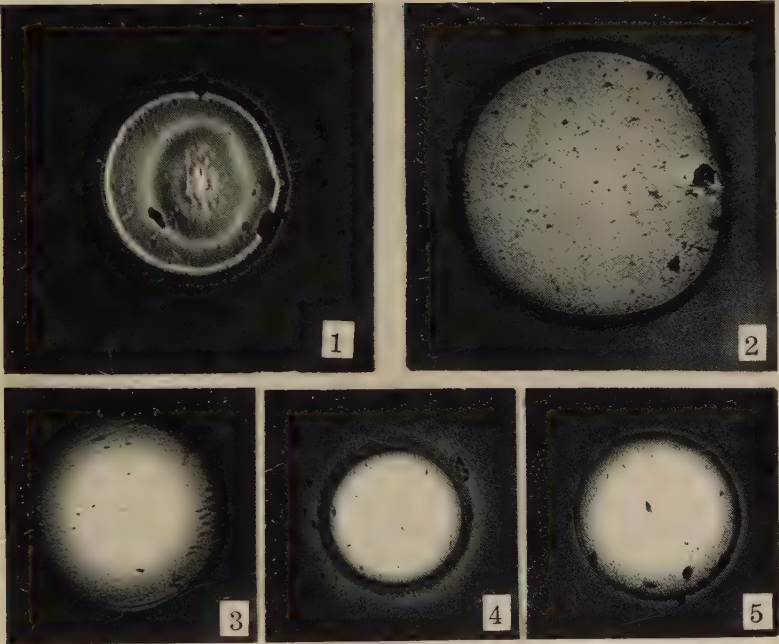
2 id. positive (+) from a normal rat (pH 6.8).

3 id. strongly positive (++) from a rat injected 10 hours before with 10 g.p. units of thyrotropic pituitary extract (pH 7).

4 id. strongly positive (++) of a rat fed on KI for 12 days (pH 7).

5 id. strongly positive (++) of a normal rat with buffer pH 2.

In all cases magnification $\times 60$.



THE EYE OF THE OWL MONKEY (NYCTIPITHECUS)

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FOUR TEXT FIGURES AND ONE PLATE (SIX FIGURES)

INTRODUCTION

Monkeys in general are essentially diurnal in their mode of life. There is one, however, the platyrrhine monkey, *Nyctipithecus*—which is strictly nocturnal in its habits. The structure of its eye, and in particular the retina, thus becomes a matter of interest. Woollard ('27) described the retina of this form and pointed out its salient features which are typical of a nocturnal animal, viz.; the absence of a macula and fovea, and the possession of a pure rod retina. In the light of his description it might seem redundant to add to our knowledge of this eye. His description, however, is not accompanied by adequate illustrations. There are other noteworthy features of this eye which he did not discuss and which merit consideration. Moreover, Woollard used his observations on the retina to support the conception that *Nyctipithecus* is the most primitive of the Platyrrhines—a deduction which seems unwarranted upon the basis of retinal structure.

Through the kindness of Professor McGregor of Columbia University, I obtained two eyes of *Nyctipithecus* which he, himself, enucleated some years ago and fixed in 10% formalin. Owing to prolonged storage in formalin the eyes were quite hard, but it was possible by slow embedding in celloidin to obtain satisfactory sections of the whole eye (including the lens) at 16μ in thickness. This did not permit detailed cytological studies of the retina, but the general features of all bulbar structures cut at this thickness and stained with iron haematoxylin and erythrosin, could be examined satisfactorily.

OBSERVATIONS

Although *Nyctipithecus* is a real monkey, the general features of its eye correspond more closely to those of the nocturnal lemuroids, *Galago* and *Nycticebus*, than to those which typify the anthropoid eye. This is not because it is a primitive form but because the eye is specialized for nocturnal existence. Comparative size relations are shown in figures 1-4 and the ratios in the table. The cornea presents a marked curvature (fig. 1) like that of *Nycticebus* (fig. 3) and occupies

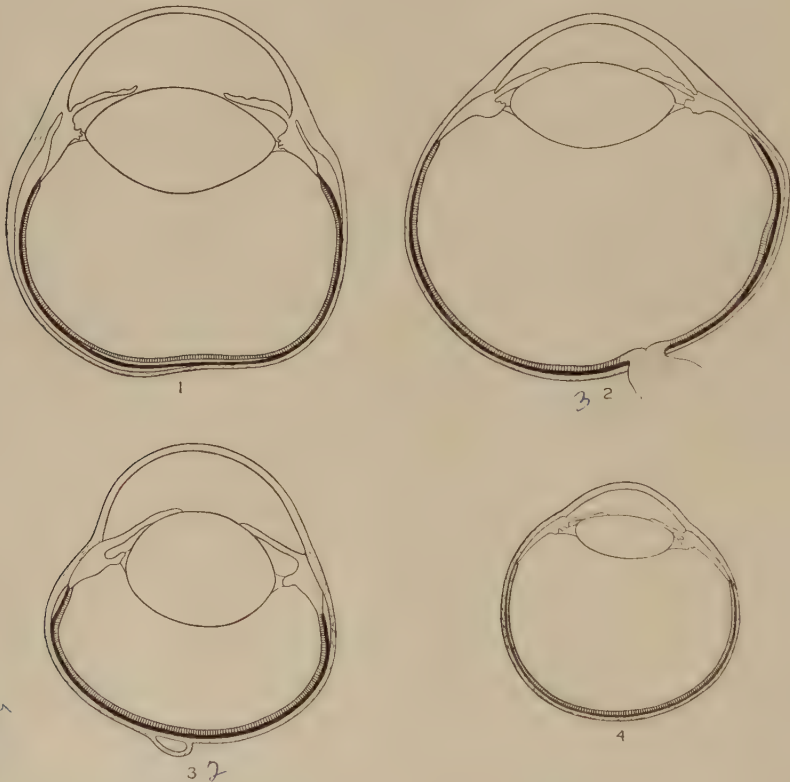


Fig. 1 Meridional section of the eye of *Nyctipithecus*. $\times 3$.

Fig. 2 Meridional section of the eye of *Nycticebus tardigradus*. $\times 3$.

Fig. 3 Meridional section of the eye of *Macacus rhesus*. $\times 3$.

Fig. 4 Meridional section of the eye of the marmoset. $\times 3$.

Table 1
1949

one-third of the perimeter of the bulb. This is in striking contrast with the diurnal marmoset where the cornea constitutes only one-fifth the perimeter of the globe—a condition very similar to that in the human eye. The lens is much larger in relation to the size of the whole eye and to the vitreous than it is in the marmoset and the rhesus monkey. This is brought out in figures 1, 2 and 4, also in the table where it is seen that the size relation of lens to vitreous is as 1:3.7, a relation which much more approaches the conditions in *Nycticebus* than in the diurnal monkeys (marmoset and rhesus).

TABLE 1

Showing ratios based upon comparative measurements of the eyes of a lemuroid and three anthropoids¹

FORM	A		B		C	
	Cornea to sclera	Cornea to whole eye	Ant. chamber to vitreous	Lens to vitreous	Ciliary muscle to lens	Ciliary muscle to whole eye
<i>Nycticebus</i>	1: 1.85	1: 2.85	1: 2.8	1: 2.3	1: 15.2	1: 79.0
<i>Nyctipithecus</i>	1: 2.00	1: 3.00	1: 3.8	1: 3.7	1: 15.0	1: 103.5
Marmoset	1: 4.10	1: 5.00	1: 15.2	1: 9.3	1: 11.0	1: 128.6
Rhesus monkey	1: 3.10	1: 4.13	1: 13.5	1: 7.1	1: 17.4	1: 283.1

¹ Measurements made from sections through the horizontal and vertical meridian.

A — Based on linear measurements in inches of eye sections at a magnification of $\times 11$.

B — Based on planimeter surface area measurements of the various structures drawn at a magnification of $\times 11$.

C — Same as B, only at a magnification of $\times 26$.

The anterior chamber is likewise large in relation to the size of the vitreous. Here again the size relations of these two structures much more approach the conditions in the nocturnal lemuroids than in the diurnal anthropoids.

The ciliary muscle in its size relation to the lens is very similar to that found in *Nycticebus* and the rhesus monkey, but when related to the size of the eye as a whole, it is found to be smaller in *Nyctipithecus* than in *Nycticebus*, but much larger than in the rhesus monkey. In general a large lens is accompanied by a large ciliary muscle, but the relationship is quite variable. Both *Nycticebus* and *Galago* have very

large lenses yet the ciliary muscle is relatively much larger in the former than in the latter. In the marmoset, where the lens is relatively small, the ciliary muscle is disproportionately large. This form, as has been pointed out previously (Detwiler, '40), appears to possess a very fine accommodating mechanism. In contrast with this situation is that found in the nocturnal Gecko where an enormous lens (v. Detwiler, op. cit., fig. 4) is accompanied by an extremely small ciliary muscle. This creature, with its large, almost spherical lens and a weakly developed ciliary muscle obviously possesses very poor powers of accommodation—if indeed it possesses any such powers at all.

The retina

My observations upon the retina of *Nyctipithecus* are in general agreement with those of Woollard ('27). There is no evidence of a fovea centralis. Cones are entirely absent and this form can be said to possess a pure rod retina (figs. 5 and 6), such as is found in the eyes of the nocturnal lemuroids (*Galago* and *Nycticebus*). In the fundus of the eye the rods are much more closely packed and attenuated than in the more peripheral areas. This is a general characteristic of all vertebrate eyes—even in such forms as lack the presence of a fovea centralis. There is no evidence of an area centralis retina such as Woollard ('25) described for the pure rod retina of *Tarsius*.

The pigment epithelial layer in *Nyctipithecus* differs from that of the nocturnal lemuroids despite the fact that in all other essentials the retina is very similar. In *Nyctipithecus* this layer is pigmented throughout, but in *Galago* and in *Nycticebus* it is devoid of pigment in the fundus of the eye and becomes pigmented only as one approaches the ora serrata. The reasons for this fundamental difference in nocturnal animals with otherwise very similar retinas is not apparent.

DISCUSSION

The main interest in the eye of *Nyctipithecus* lies in the fact that it possesses those typical structural features which characterize nocturnal vision. Apparently *Nyctipithecus* is the only known monkey which is nocturnal in its habits. Its eye has been found to bear a striking resemblance to those of the nocturnal lemuroids (*Galago* and *Nycticebus*) described previously (Detwiler, '39, '40), and does not exhibit the specific features which characterize the eyes of the diurnal marmoset and rhesus monkey, also hitherto described (op. cit.). This is evident from the relations given in the table and in the differences brought out in the figures.

Woollard ('27) who described the retina of this form as a pure rod retina—devoid of cones and a fovea—says “*Nyctipithecus* is no exception to the rule that a true macula and fovea are only found when cones form the chief constituent of the sensory epithelium of the retina. These observations add support to the conception that *Nyctipithecus* is the most primitive of the *Platyrrhines*.” The first part of his statement is true, but it is apparent that the lack of cones and a fovea can in no manner be used as an argument in support of the thesis that *Nyctipithecus* is the most primitive of the *Platyrrhines*. Apparently, some authorities regard the marmoset as a more primitive form than *Nyctipithecus*, yet its retina not only predominates in cones, but it possesses also a typical anthropoid fovea (figs. 9 and 10). Neither the presence nor absence of a fovea and cones can be used as characters to help pigeon-hole any animal in the evolutionary scale. Although the eyes of vertebrates are built upon a common generalized plan, nature has deviated considerably from this plan in many instances, and the various specializations which one encounters carry no evolutionary significance. Especially is this true of the fovea which is found in many salt water fishes, certain reptiles, birds, and most anthropoids, but which is absent in fresh water fishes, amphibians, many

reptiles (particularly the chelonians) and mammals. Eyes with pure rod retinas are specializations and are correlated with nocturnal habits. Those with pure cone retinas are equally specialized for diurnal vision at high intensities of light. Foveas constitute mechanisms for increased visual acuity, be they present in fishes, lizards, birds, monkeys or man. Pure rod retinas are adaptations for nocturnal vision whether they occur in lizards, bats, rats, lemurs or monkeys.

SUMMARY

1. The eye of the owl monkey, *Nyctipithecus*, possesses the structural features characteristic of nocturnal vision. The cornea which exhibits a marked curvature constitutes approximately one-third the perimeter of the globe. The anterior chamber in relation to the vitreous is about four times as large as in the eye of the marmoset and of man. The lens is also relatively much larger than in the diurnal anthropoids hitherto studied (marmoset and rhesus monkey). Although *Nyctipithecus* is a primitive monkey, the relative size proportions of the various eye structures simulate much more closely those of the nocturnal lemuroids (*Galago* and *Nycticebus*) than those of the diurnal monkeys (table and figs. 1-4).

2. The retina is devoid of a fovea and of cones. In its structural make-up it resembles closely that of the nocturnal lemuroids. The rods in the fundus are attenuated and closely packed. There is no evidence of any specialization such as an area centralis retina. The pigment epithelial layer possesses a moderate amount of pigment which is in contrast with this layer in *Galago* and *Nycticebus* where epithelial pigment is lacking except near the ora serrata.

3. Comparisons in the form of meridional sections and size ratios are given of the various eye structures in the nocturnal lemuroid, *Nycticebus tardigradus*; the nocturnal *Platyrrhine* monkey, *Nyctipithecus*; and the diurnal monkeys, *Hapajacus* and *Macacus rhesus*.

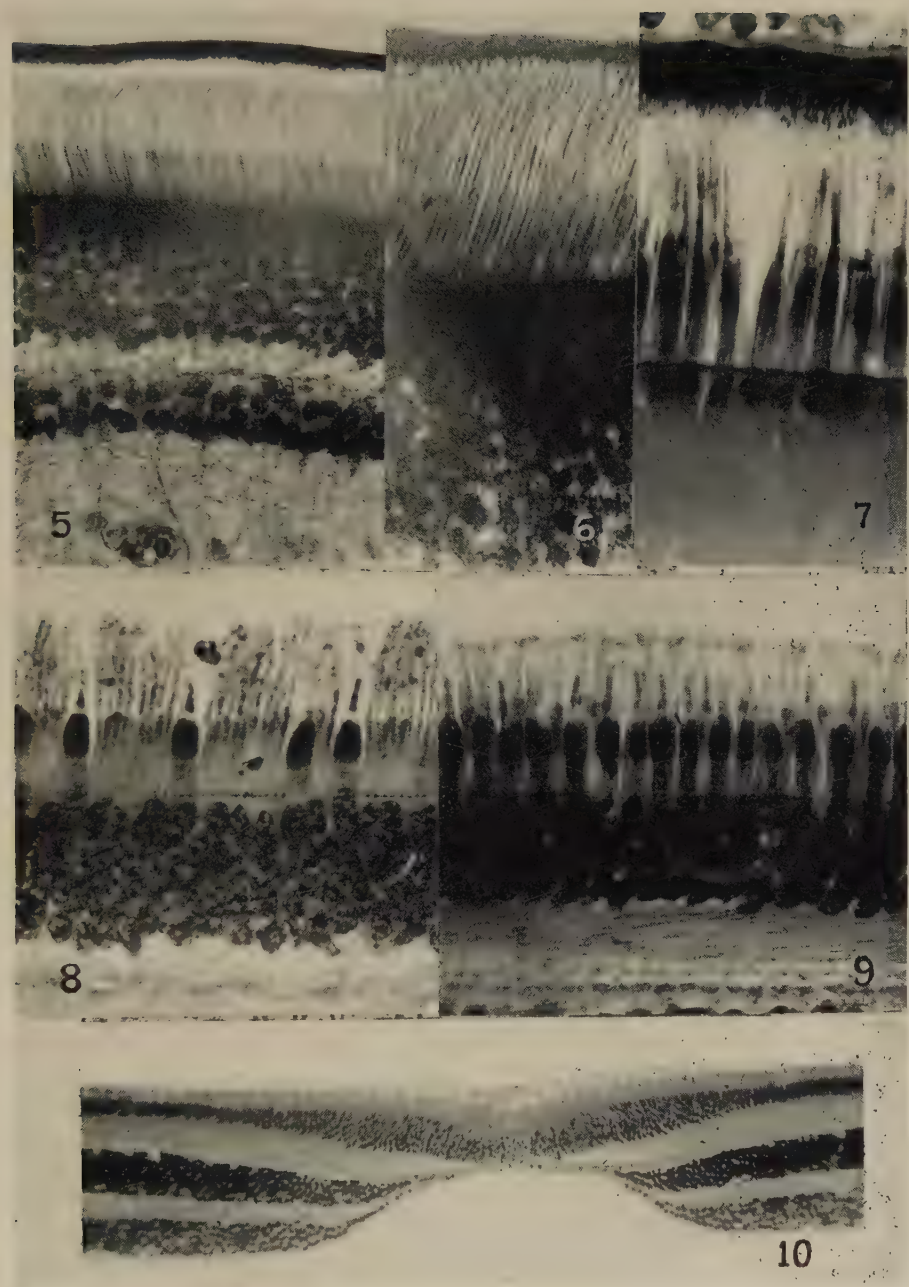
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PLATE 1

EXPLANATION OF FIGURES

- 5 Photomicrograph of the the retina of *Nyetipithecus*. $\times 450$.
- 6 Photomicrograph showing rod visual cells of *Nyetipithecus*. $\times 630$.
- 7 Photomicrograph of rhesus monkey eye in the parafoveal region, showing slender elongated cones and rods. $\times 630$.
- 8 Photomicrograph of rhesus monkey eye near the ora serrata, showing sparse broad cones and numerous rods. $\times 630$.
- 9 Photomicrograph of the marmoset retina in the parafoveal region showing both cones and rods. $\times 630$.
- 10 Photomicrograph of the marmoset fovea. $\times 100$.



THE STRUCTURE OF THE OVARY OF THE HUMPBACK WHALE (MEGAPTERA NODOSA)

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ONE TEXT FIGURE AND THREE PLATES (FOURTEEN FIGURES)

The present paper deals with the structure of the ovary of the humpback whale (*Megaptera nodosa*). The material was collected by Lt. T. R. Midtlyng, U. S. C. G., who was attached to the whaling factory reduction ship S.S. Frango, operating in Shark's Bay, Western Australia, in 1938. Mr. Charles E. Jackson, Acting Commissioner of the Bureau of Fisheries, kindly placed the material at our disposal.

MATERIAL AND METHODS

The ovaries from sixteen whales were removed and placed in 10% formalin. After they had been shipped to the laboratory, they were examined and freehand cuts were made through any grossly visible surface markings. Photographs were taken of the cut surfaces. Representative blocks were embedded in celloidin and the sections were stained either with hematoxylin and eosin or with the azan method.

The diameter of ova and follicles in stained preparations was measured with a micrometer eye-piece. In addition, the jelly representing the fixed follicular fluid was scooped out of certain follicles and the egg was identified with the aid of a binocular dissecting microscope. Direct photographs were then prepared and the ovum diameter was computed from the image size and the magnification factor.

OBSERVATIONS

The external surface of the ovary presents a convoluted appearance. Sections through the ovaries show that these convolutions consist of a central core of connective tissue containing blood vessels and of a mantle of ovarian cortex which measures 1 to 2 cm. in thickness (figs. 6 to 10). The folds of the cortex contain either follicles or corpora lutea.

The shape of the ovary is irregular, but usually it is elongated in one diameter (see figs. 6 to 8). The greatest diameter ordinarily is 10 to 15 cm., while the other diameters are approximately half as large. The weight of the two ovaries ranges from 0.5 to 1.5 kg. Single ovaries may weigh as much as 1.4 kg.

The ovaries from all the mature animals contain many small follicles (diameter 1.0 to 1.5 cm.). In addition, larger follicles, located near the surface, are present in three specimens. The diameters of these large follicles are from 3.5 to 5.0 cm. For the specimen in which the 3.5 cm. follicle was found, a notation had been made that the animal was in heat and had been killed in the company of a mature male.

Microscopically, the follicles are similar, except for size, to the graafian follicles of other mammals. The one at the bottom of figure 2 is the 3.5 cm. follicle, mentioned in the preceding paragraph, from the ovary of an estrous whale. A section through the ovum and germinal hillock of this follicle is shown in figure 3. The granulosa layer is quite narrow, as if it had been stretched during the enlargement of the follicle. Cavitation within the cumulus oophorus is seen. This condition is found in the preovulatory follicles of other mammals (Myers, Young and Dempsey, '36), and is therefore a finding which supports the conclusion that this follicle is in the mature, estrous condition.

The size of the whale ovum is rather small in comparison with ova from other species (compare Hartman, '29). Figure 5 is a photograph of an ovum which was scooped out of a 1.5 cm. follicle, together with the follicular jelly and the granulosa elements. The ovum, after identification with a dissecting

microscope, was placed on a glass slide, covered, and photographed. Computation of the diameter from image size and magnification factor gave a value of 130μ . It is thought, however, that this value is somewhat large, because of compression of the egg between the slide and the cover slip. Measurements of the eggs from large follicles in stained material gave values of 90 to 100μ . Since there is shrinkage in stained preparations, the true value probably is somewhere between 100 and 130μ .

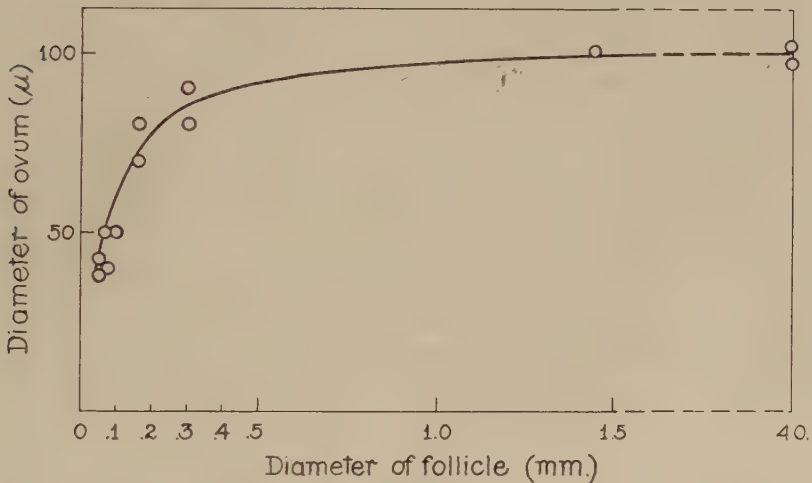


Fig. 1 The relationship between follicular and ovum diameters in the humpback whale.

The relationship between ovum and follicular diameters is shown in figure 1. It can be seen that the ovum attains its maximum size while the follicle is still small. Indeed, the ovum is mature, as judged by size, at the time the follicle has a diameter of 1 mm., whereas the size of the resting follicle is 1 to 1.5 cm. and the mature follicle 3.5 to 5 cm. The antrum forms when the follicle has a diameter of about 0.3 mm. and consequently the ovum has nearly completed its growth while the follicle is still solid. These facts are in agreement with similar findings in other mammals (compare Parkes,

'31), but are rendered more striking because of the large final size which is attained by the whale's follicles.

Corpora lutea are present in the ovaries of all the mature animals. They are of varying size and appearance, and are illustrated in figures 4 and 6 to 10. The early corpora lutea, shown in figures 4, 6 and 7 (cl_1), are somewhat flattened, collapsed structures measuring 3 to 4 cm. in greatest diameter. The granulosa layer has begun to hypertrophy, resulting in the formation of folds surrounding a central cavity which contains a fresh plasma clot tinged with blood (figs. 6, 7 and 11).

Older corpora lutea are larger (5 to 6 cm.) and are spherical as a result of continued hypertrophy of the luteal tissue. The luteal tissue is cream colored at this stage. Figure 8 (cl_2) illustrates one of these mature corpora lutea, with the persisting central cavity filled by a fibrinous clot (fig. 12).

Successive later stages of corpora lutea are illustrated in figures 9 and 10. The color of the luteal tissue has gradually changed until at the stages here referred to the corpus luteum is a bright lemon yellow. The central cavity has been slowly invaded by connective tissue. This process begins by the appearance of strands of fibroblasts, accompanied by numerous macrophages, which migrate in from the adjacent luteal tissue. The connective tissue fibers, constituting a reticulum, stain blue with azan, in contrast to the bright red color of the residual fibrin mass. This leads eventually to the complete replacement of the fibrin of the previous stages by organized connective tissue (figs. 9, 10 and 14). Finally a zone of hyaline degeneration forms at the border between the central core of connective tissue and the surrounding luteal tissue. At the same time the connective tissue of the theca externa, in the spaces between the folds of luteal tissue, increases in amount and density, with the result that the luteal cells become arranged in characteristic cords which are separated by septa of fibrous tissue as shown in figures 10 and 14. The individual luteal cells have become smaller at this stage.

Subsequent regression of the corpus luteum is apparently slow. The luteal tissue becomes more and more shrunken and takes on a glassy translucent quality exhibiting a pale yellow color, whereas the central connective tissue core becomes increasingly dense and hyaline and assumes a white color. Figure 7 shows, in addition to a recently formed corpus luteum (cl_1), one of these older, regressing structures surrounded by a capsule of connective tissue (cl_3).

The vascular supply of both follicles and corpora lutea is similar to that found in these structures in other mammals. There are no blood vessels in the granulosa layer of the follicles. Vessels of capillary diameter are found in the theca of the follicles. After ovulation this wreath of thecal vessels grows into and constitutes a vascular network within the enlarging luteal tissue. It is apparent from examination of one section that the blood vessels which penetrate the corpora lutea remain exceedingly small despite the immense size of the mature luteal body and the excessive diameter of the luteal zone. Moreover, the afferent vessels, lodged in the connective tissue septa between the luteal convolutions are likewise excessively small and slender. In view of the disproportion between the large corpus luteum and the small caliber of the vessels supplying it, we are led to assume that the precapillary arterioles and capillaries of the whale's luteal body must be unusually long. Figure 13 shows vessels of capillary diameter which are found within the luteal tissue.

The ovarian medulla, on the contrary, contains many blood vessels of large caliber, which are embedded in dense stroma. The arteries appear to follow a spiral course. Figure 10 presents the pattern of the vessels in the medulla. The relatively large size of the medullary vessels, on the one hand, and the delicacy of the luteal vessels, on the other, suggest that there must be an abrupt narrowing down or constriction where the former give rise to the latter. Such an abrupt narrowing of the medullary arteries at the border of the cortex in the manner of pipettes or of nozzles has recently been described in the human and rabbit's ovary by Dabelow ('39).

DISCUSSION

In general form and appearance the ovarian structures of the humpback whale are similar, except for size, to the same structures in the ovaries of other mammals. Moreover, the cyclic succession of follicles and corpora lutea occurs in a manner entirely comparable, with one exception, to that seen in other mammalian forms. This exception is the extremely slow rate of regression of the senile corpora lutea in comparison to their rate of involution observed in other forms. The consequence of this slow regression is that many scars of old corpora lutea are always present in the ovary. Indeed, these regressing corpora lutea persist for so long a time that MacIntosh and Wheeler ('29), in discussing their presence in the ovaries of blue and fin whales, conclude that they are never completely resorbed. Matthews ('37) agreed with this conclusion after studying the ovaries of humpback whales and accepted the implication that a record of the total number of past reproductive cycles may be found in the ovaries of any whale. Whether or not this statement is entirely correct, it is perfectly clear that the scars of old corpora lutea persist for an exceedingly long time, since as many as thirty have been found in a single pair of ovaries.

It seems reasonable that these remnants of corpora lutea persist for such long periods because of the extreme density and extensive hyalinization of the connective tissue which forms their stroma and central cores. Initially, the corpora lutea are so large that, although they regress steadily in size, resorption of their elements is a protracted process. In this prolonged involution they surpass the corpora lutea of other animals.

Interest attaches to the mode of formation of the large hyaline core which is formed within the corpus luteum, and which constitutes the principal element of the regressing luteal body. It is well known in pathology that hyaline degeneration is frequently associated with a deficient blood supply. Hence the thought lies close at hand that the relatively

large size of the corpus luteum in the whale may impose certain limitations and restrictions on its vascular supply, with the result that it undergoes marked hyalinization.

Clark ('00) and Andersen ('26) have described the blood supply of the corpus luteum of man and sow. According to these authors, arterioles and venules grow into the corpus luteum from the follicular wreath. These capsular arterioles and venules follow the septa or folds of thecal connective tissue which indent the surface of the corpus luteum, and finally give off capillary loops which penetrate into the growing luteal tissue. Reasoning by analogy, since the indenting septa of the whale's large corpus luteum are exceedingly deep, it follows that the slender, small-calibered arterial vessels observed within them must be relatively long. Similarly, since the folded mantle of luteal tissue forming the whale's corpus luteum is excessively thick (figs. 12 and 14) and since the blood vessels penetrating it are solely capillaries, it may be assumed that the capillaries are also unusually long. Thus one of the principal peculiarities of the architecture of the whale ovary is the relatively marked lengthening of the smallest arterioles and the capillaries supplying the corpora lutea.

There are well-known physiological observations that resistance to flow is greater in long than in short blood vessels of the same diameter. In consequence, blood flow is less through long vessels unless compensated for by adjustments of pressure or diameter. These adjustments have, however, definite limitations, because increased capillary pressure entails excessive fluid loss from the capillaries, whereas increased capillary diameter can function to regulate blood flow only if the resistance in the capillary segment is greater than that of the arteriole. It may be assumed, therefore, that there is a limit to the possible lengths that capillaries and arterioles may attain. This limit appears to have been reached in the whale's corpus luteum judging by the unusual thickness of the luteal tissue, as well as by the presence of a large

central cavity which does not become filled by the hypertrophying luteal cells. Moreover, the extensive fibrin clot filling the central cavity becomes replaced gradually by an excessive amount of hyaline connective tissue almost devoid of blood vessels. Thus we believe that the blood supply of the whale's corpus luteum is subjected to anatomical and functional limitations which result in excessive fibrosis and hyalinization accompanied by delayed involution of the luteal scars.

SUMMARY

The ovary of the humpback whale (*Megaptera nodosa*) is a large structure weighing from 0.5 to 1.5 kg., the surface of which is thrown into folds, clothed by a lamina of cortex which is 1 or 2 cm. in thickness.

The ovarian follicles are similar, except for their large size (1 to 5 cm.), to the same structures in the ovaries of other mammals. The ova, on the other hand, are relatively small, possessing diameters of from 100 to 130 μ .

The corpora lutea are excessively large (3 to 6 cm.) and, in the mature condition consist of a zone of lutein tissue surrounding a large central cavity in which an original fibrin clot has undergone complete fibrosis and hyalinization. On account of the marked fibrosis and hyalinization, involution of old corpora lutea is extremely retarded, and many luteal bodies may be found in the ovaries of mature animals.

We conclude that the large size of the whale's corpus luteum imposes certain anatomical peculiarities and physiological limitations on its vascular supply. The luteal arterioles and capillaries are apparently unusually long. The functional restrictions in consequence of the lengthening of these vessels make it impossible for the luteal tissue ever to fill the entire cavity of the luteal body. These limitations result also in nearly complete avascularity of the center of the corpus luteum, associated with excessive fibrosis and hyalinization.

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PLATE 1

EXPLANATION OF FIGURES

2 Ovary from an estrous whale, showing a large 3.5 cm. follicle at the bottom of the figure. $\times \frac{1}{2}$.

3 Section through the ovum from the follicle shown in figure 2. The granulosa layer is thin. Cavitation has occurred in the germinal hillock. The nucleus of the ovum is still in the resting stage. $\times 48$.

4 Ovary showing a recently ruptured follicle at the lower left corner of the figure (cl₁). Many 1 to 2 cm. resting follicles are present. $\times \frac{1}{2}$.

5 Photograph of the ovum which was scooped out of one of the resting follicles shown in figure 4. The diameter of this ovum was 131 μ . $\times 230$.

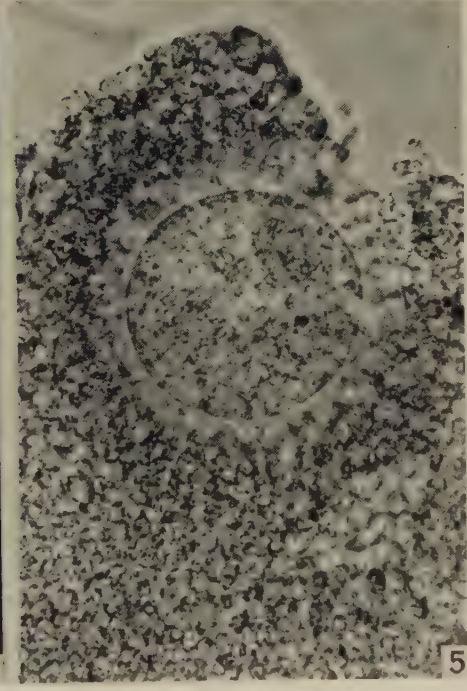
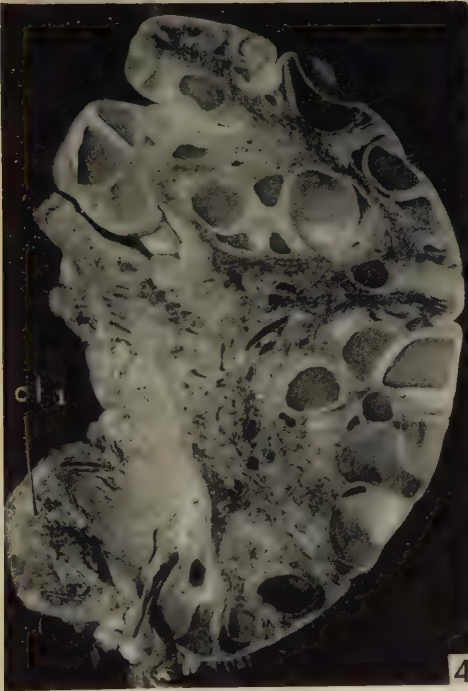


PLATE 2

EXPLANATION OF FIGURES

6 Ovary containing an early corpus luteum (cl_1). A section through this corpus luteum is shown in figure 11. The surface of the ovary is folded or convoluted as a result of overgrowth of the cortex. $\times \frac{1}{2}$.

7 Ovary containing a recent, organizing corpus luteum (cl_1) and an older, hyaline, regressing luteal structure (cl_3). $\times \frac{1}{2}$.

8 Ovary containing a large, organizing corpus luteum (cl_2). A section through this structure is presented in figure 12. The central cavity contains a clot of fibrin at this stage. $\times \frac{1}{2}$.

9 Ovary containing a corpus luteum older than that shown in figure 8. The structure has attained its greatest size at this stage. A hyalinized ring surrounds the central core of connective tissue. A section through this corpus luteum is shown in figure 14. Large blood vessels can be seen in the medulla of the ovary and in the capsule which surrounds the corpus luteum. $\times \frac{1}{2}$.

10 Ovary containing a regressing, partly hyalinized corpus luteum. The center has become organized into a dense core of connective tissue. The septa between the folds of luteal cells also have become fibrotic. Large blood vessels can be seen in the medullary region of the ovary. $\times \frac{1}{2}$.

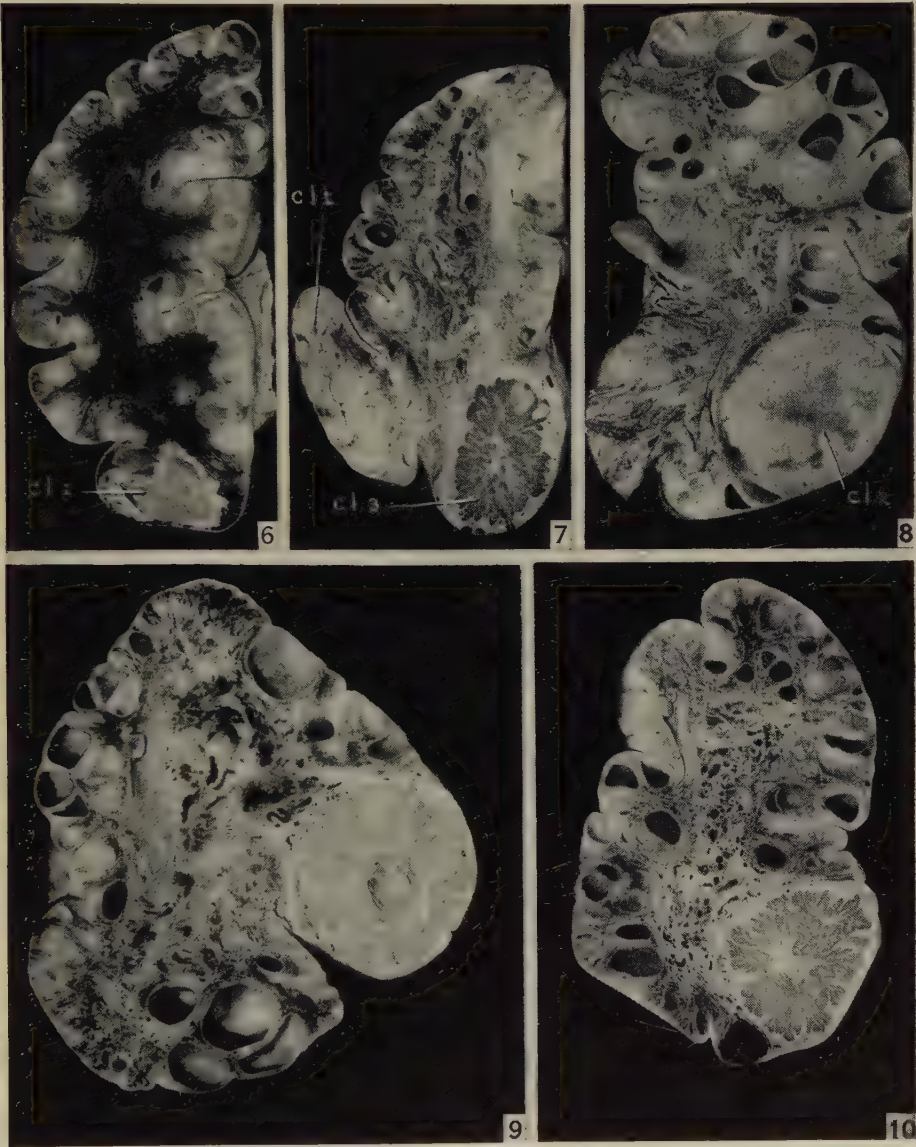


PLATE 3

EXPLANATION OF FIGURES

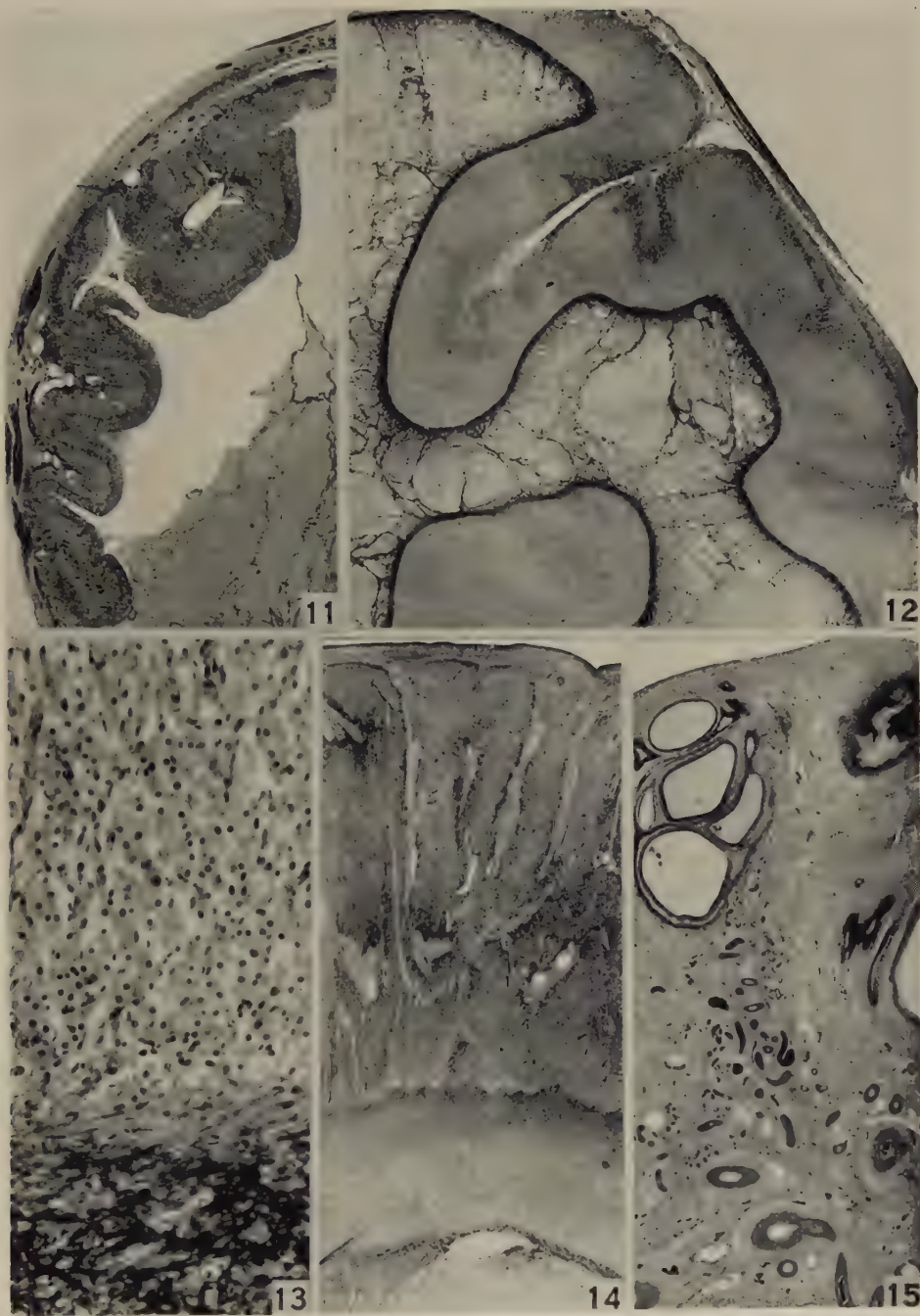
11 Section through the early luteum shown in figure 6. Folds have begun to form in the hypertrophying granulosa layer. Large blood vessels can be seen in the capsule surrounding the corpus luteum. The follicular fluid in the central cavity contains strands of fibrin. $\times 4$.

12 Section through the organizing corpus luteum shown in figure 8. The layer of luteal cells has become thicker and the infolding process has continued. Small arterioles can be seen running along the fold shown in the upper part of the figure. The central cavity is now filled with a network of reticulum fibers and fibrin. The fibers contiguous to the luteal cells stain blue with the azan stain (reticulum) while those farther toward the center stain red (fibrin). $\times 4$.

13 Higher power photograph of the corpus luteum shown in figures 8 and 12. The large luteal cells and the capillary blood vessels coursing between them can be seen. The connective tissue of the theca interna is shown at the bottom of the figure. $\times 150$.

14 Section through the wall of the corpus luteum shown in figure 9. The connective tissue septa, continuous with the outer capsule, are shown. Small blood vessels may be seen within these septa. The core of the corpus luteum comprising a loose fibrous center surrounded by a dense hyaline zone is seen at the bottom of the photograph. $\times 4$.

15 Section through an ovary showing the compactness of the stroma and the large size and abundance of the blood vessels in the medulla. $\times 4$.



AN ABERRANT FUNCTION OF OMENTAL LYMPHATICS IN OMENTAL- JEJUNAL ADHESIONS

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THREE FIGURES

A considerable amount of study has been given to the physiological importance of the blood vascular system in the relief of congestion through the establishment of omental adhesions. The similar role, played by lymphatics, has received relatively little consideration. Although we demonstrated lymphatic anastomoses in adhesions between the omentum and jejunum in dogs by means of India ink injections (Simer and Webb, '37), it does not follow, necessarily, that these connections actually function as a collateral pathway. Continuing our experiments on the newly formed lymphatic connections of omental-jejunal adhesions, we have tried to determine to what extent they assume the duties of the intestinal lacteals in the drainage of chyle from the jejunum.

Some aspects of problems related to the physiology of lymph vessels in omental-jejunal adhesions have been considered previously. During a study of regeneration of mesenteric lymph nodes after their excision, Meyer ('06) accidentally produced an adhesion between the small intestine and the omentum in a dog. Seven days later he found little pockets of chyle within the adhesion. He injected Prussian blue into the intestinal wall, but did not see dye in the omental lymphatics. Coffin ('06) demonstrated newly formed lymphatics growing into granulation tissue from the subserous layer of the small intestine 7 to 10 days after the gut had been partially

exposed through a wound in the skin of the abdomen. This experiment shows that intestinal lymphatics will sprout into adjacent tissue. In an explanation of the probable causes of gastric and duodenal ulcers, Braithwaite ('23) assumed that infectious substances are transported by the omental lymphatics from the region of an inflamed appendix. His evidence was limited to the demonstration of lymph channels in the omentum and their termination in the duodenal lymph node. Connections between the lymphatics of these organs were not observed. Macguire ('35) concurred with the assumption of Braithwaite but presented no additional evidence. Attempting to explain his success in relieving symptoms of elephantiasis in two patients by establishing adhesions between the omentum and the anterior abdominal wall, Dick ('35) postulated that an anastomosis of the lymphatics had been established. His endeavors to display such pathways in experimental adhesions in rabbits were unsuccessful.

We performed aseptic laparotomies on seventeen dogs, anesthetized with nembutal. In all animals the mesenteric blood and lymph vessels supplying a segment of the jejunum about 10 cm. in length were ligated near the intestine. The serosa of this segment was scraped with a scalpel and the free end of the omental sac attached to it with two or three sutures which penetrated the musculature. Best adhesions resulted when the intestinal wall was scraped as deep as the longitudinal muscle layer. After closure of abdominal incisions, all dogs passed through a normal period of convalescence. The period of time between the operation and post mortem examinations varied from 6 days to 245 days, when we found fourteen of the seventeen adhesions still intact. In the three animals where the omentum had become detached from the jejunum, the times elapsing since operation were 83, 85, and 189 days. Our experience indicates a tendency for self healing of such adhesions by the eightieth day following the operation.

A test of the ability of the lymphatics in the adhesions to carry chyle from the jejunum to the omental and duodenal lymph nodes was accomplished by fat feeding. The animals

were divided into three groups, each of which received a different meal preliminary to the termination of the experiment. The four dogs of group 1 were fed unstained fat in the form of cream, butter, or fat meat 4 to 5 hours before each was killed. Gross examination of their peritoneal cavities showed many distended lymphatics, appearing as white threads accompanying the blood vessels in the mesentery. In two dogs of this group, the omentum had become free from the jejunum, but in the other animals, a good adhesion remained. A careful examination failed to show white lymph channels in the adhesions of the adjoining omentum. Although this observation caused no surprise in view of the extreme difficulty one encounters in attempting to demonstrate lymphatics in normal omental tissue, it emphasized the necessity for employing some method whereby these channels could be delineated more distinctly.

The feeding of colored fat seemed to offer a solution to the problem of identifying lymph vessels in adhesions. Lemon and Higgins ('29) reported that all mesenteric lymphatics, the cisterna chyli, and the thoracic duct of dogs were colored pink a few hours after a meal of fat stained with Sudan III. Accordingly, our six dogs of group 2 were treated in the manner outlined by these investigators. They were fed a mixture of ground meat and melted butter or cream, stained with 50 mg. of Grüber's Sudan III. At post mortem examination 2 to 5 hours later, we found to our dismay that the lacteals were white and no colored material appeared in any part of the lymphatic system, although deeply stained chyme could be seen through the intestinal wall. Having failed in attempts to demonstrate lymphatics in omental-jejunal adhesions by the method indicated, we prepared pieces of this tissue for microscopic examination. They were fixed in 10% formalin and embedded in gelatin according to the method of Gaskell ('12). Frozen sections, 25 to 100 μ thick, were made from blocks of omental tissue containing fat strips which led directly from an adhesion. Some of the unstained sections were mounted in water, glycerin, or glycerine jelly and studied

microscopically. No trace of color appeared in any lymph channels of these preparations. Other sections were stained with an alcoholic solution of Sudan III and mounted in a similar manner. In most of them, the lymphatics were clear or faintly tinted in contrast to the deep orange color of the surrounding omental fat cells (fig. 1, A). Stained sections from two of the animals contained lymphatics that were distinctly colored (fig. 1, B) indicating that chyle had reached them from the jejunum.

As a result of our experience, we must conclude that feeding dogs fat stained with Sudan III does not give uniform results, because we could not verify the observations of Lemon and Higgins. Similar difficulty was experienced by King, Arnold and Church ('22), as evidenced by their statement, "Attempts were made to color the central lacteal by feeding the animal (dog) fats stained with sudan III and also Scharlach R, but in not a single case was there the slightest evidence of the stain having found its way into the central lacteal". An attempt was made to check the possibility of staining the chyle in lacteals of another laboratory animal. Eight rats were fed fat stained with Grüber's Sudan III and examined 2 to 4 hours later, but in no case was color discernible in their abdominal lymphatics. In an extensive article on fat feeding, Gage and Fish ('24) comment upon the confusing results obtained by Sudan III and Sudan IV stains manufactured by different companies, and they recommend National Aniline Company dyes for uniform and satisfactory results. Before employing these dyes in studies of omental lymphatics, we tested their efficacy to stain chyle in two rats and one cat, obtaining only a faint tint in the rats but an intense coloration

Fig. 1 Photomicrographs of sudan III stained frozen sections of omental fat strips containing lymph vessels leading from omental-jejunal adhesions. The animals were fed a fat meal 6 hours before death. A, lymph vessels clear, indicating that the lymphatics of the omentum and jejunum were not connected. B, a large lymph vessel filled with stained chyle, showing that a connection was established. Note contrast in the appearance of the contents of lymph vessels in A and B.

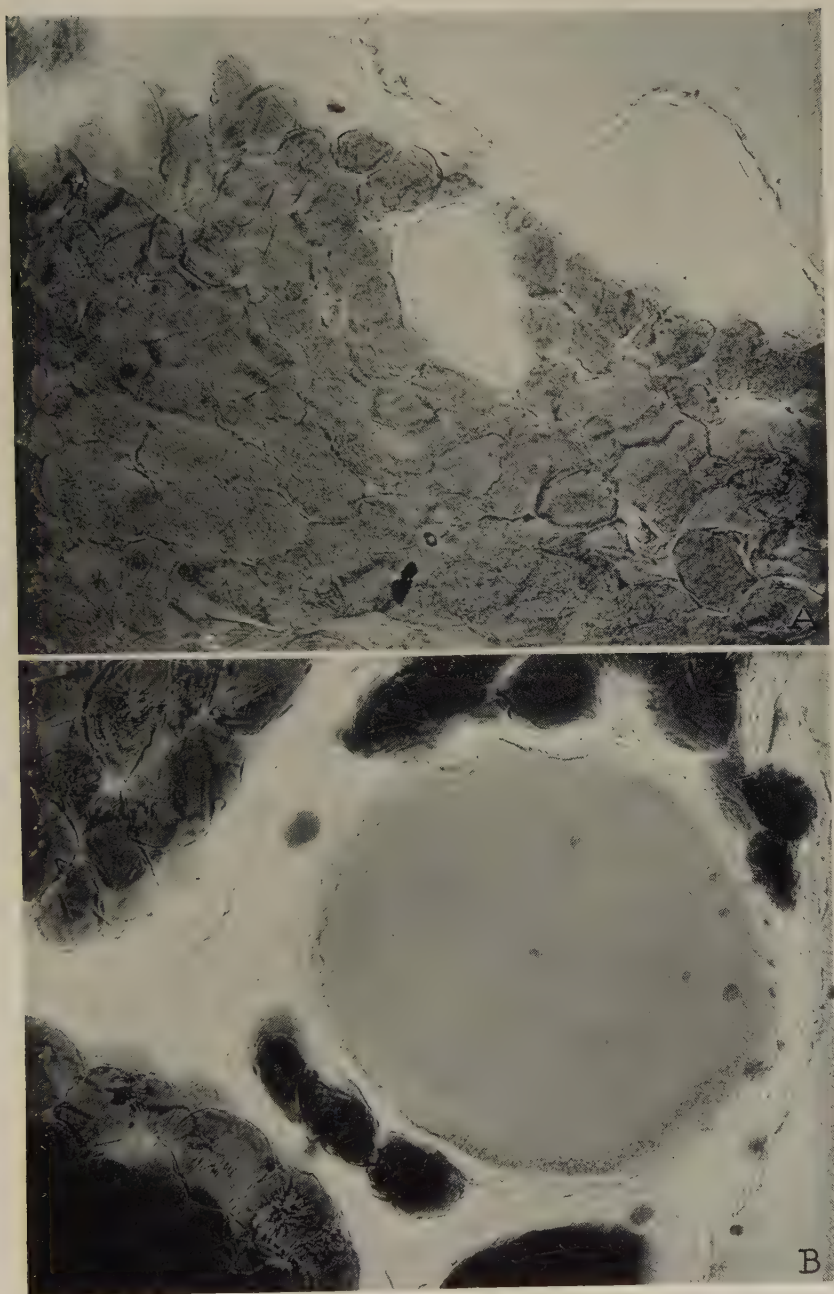


Figure 1

in the mesenteric lymphatics, cisterna chyli and thoracic duct of the cat.

Group 3, containing 8 dogs, was fed ground meat and butter stained with Sudan IV (National Aniline Company). When the animals were examined at autopsy 4 to 7 hours after feeding, the contents of the mesenteric lymphatics in six of them were colored varying degrees of pink. The failure of the dye to stain the chyle in two animals can be explained by variations in our usual feeding routine. One dog had eaten only a portion of the prepared meal and had vomited most of it. The second failure likely resulted from a poor staining of the fat meat which was fed. In this case, the dye was mixed with cold meat and fed directly, whereas the usual procedure was to add dye to melted butter and then mix this evenly stained butter with ground meat.

Good omental-jejunal adhesions were present in five of the six animals which reacted positively to Sudan IV. In two dogs, there were distinct pink lines extending from the jejunum to the omental and duodenal lymph nodes. These nodes were engorged with colored chyle. We removed the stomach with the attached omentum and its jejunal adhesion and fixed this tissue in 10% formalin. Blocks of omental fat strips containing colored lymph channels were prepared for frozen sectioning in the manner previously described. There was no difficulty in recognizing the pink color of the chyle-filled channels when sections were mounted in water (fig. 2, A), but when they were mounted in glycerine or glycerine jelly, the color tended to fade. Of the ordinary mounting media, damar was the most acceptable for temporary preparations (fig. 2, B). Because of the instability of our preparations, permanent records were made by photographing freshly mounted sections

Fig. 2 Photomicrographs of frozen sections of omental fat strips leading from omental-jejunal adhesions. The dogs received a meal of fat stained with Sudan IV (National Aniline Company) 6 hours before death. The lymphatics of each fat strip are filled with colored chyle. A, section mounted in water. Kodachrome positives of this section show definite coloration of the chyle in lymph vessel. B, a more permanent mount of a section in damar. Granular content of the chyle filled lymph channel is quite distinct. Surrounding fat cells are clear.

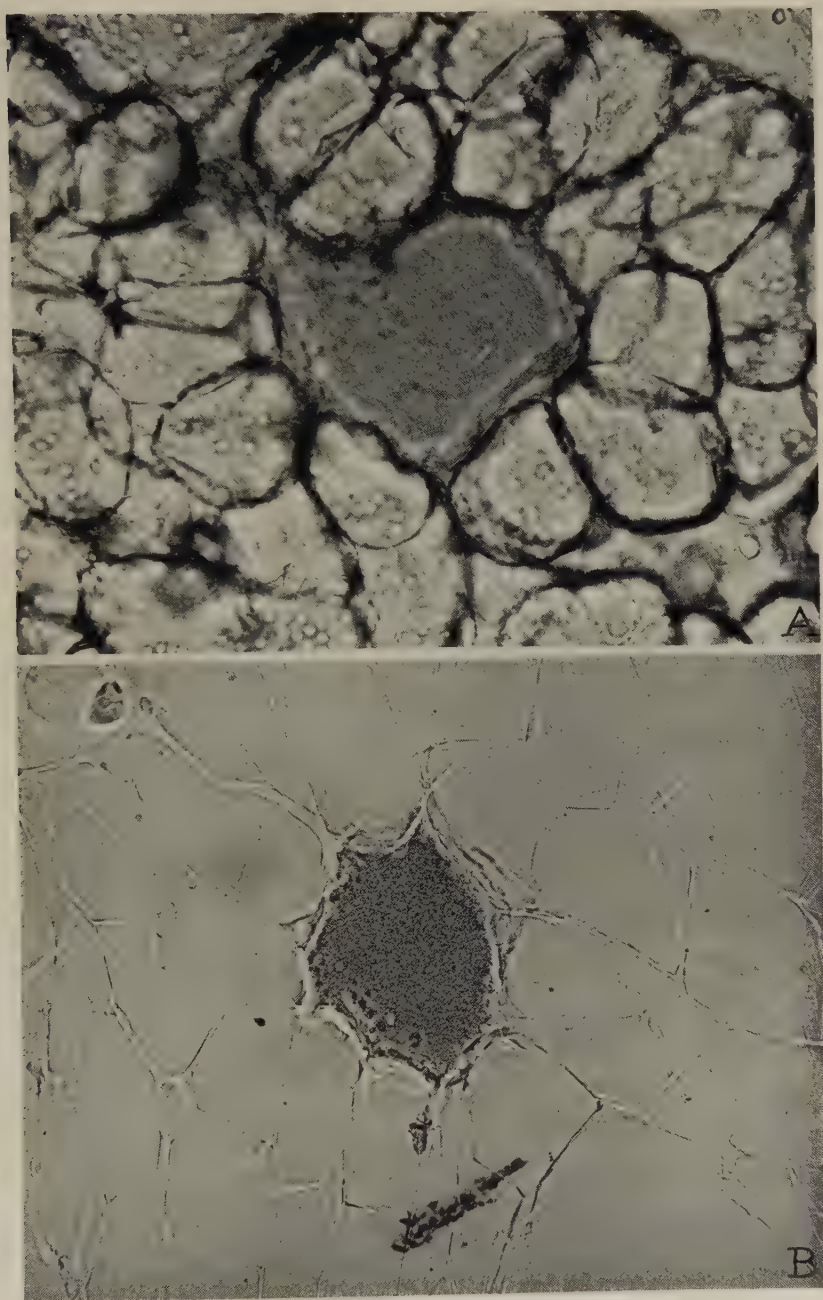


Figure 2

with Kodachrome film. These were demonstrated at a recent meeting of the American Association of Anatomists (Simer and Webb, '40). Photographs of omental fat strips containing lateral lymphatics filled with colored chyle are shown in figure 2. These channels lie near the mesothelial surface of the omentum and were visible in macroscopic study. Other vessels of this type lie deeper and are not visible upon gross examination. Our success in identifying stained chyle in these sections leads us to disagree with Gage and Fish who emphatically state that physiological stain in chyle is useful for naked eye observation only, and that it is no help for microscopic work. An examination of frozen sections stained with Sudan IV confirmed the presence of chyle in omental lymphatics leading from the jejunal adhesion, presenting a picture similar to that shown in figure 1, B.

An interesting incidental observation concerned the coloration of the duodenal nodes in the animals of group 3. In those instances where we could not demonstrate functional omental lymphatics from the adhesion, the node was colored only at the duodenal pole. Color-filled lacteals could be traced from this point to the duodenum near its junction with the pylorus. When we could clearly identify colored chyle in lymphatics of the omentum, both poles of the node were stained. This indicates that lymph from an afferent vessel is restricted to a somewhat limited area in its passage through a node.

SUMMARY

1. Omental-jejunal adhesions were produced in seventeen dogs by surgical means under aseptic conditions. At autopsy 6 to 245 days after operation, fourteen adhesions were intact. The tendency for the omentum to separate from the intestine was more pronounced as the time interval increased.

2. Newly formed lymphatic connections in adhesions between the jejunum and omentum could not be demonstrated grossly after animals had been fed uncolored fat. Attempts to stain chyle by feeding fat colored with Grübler's Sudan III were unsuccessful. However, the presence of fat was verified

in some of the omental lymph vessels by microscopic examination of frozen sections stained with Sudan III.

3. Physiological staining of chyle was accomplished by feeding fat colored by Sudan IV (National Aniline Company). Omental lymphatics leading from the adhesions to omental and duodenal nodes were filled with chyle and appeared grossly as meandering pink lines near the surface of fat

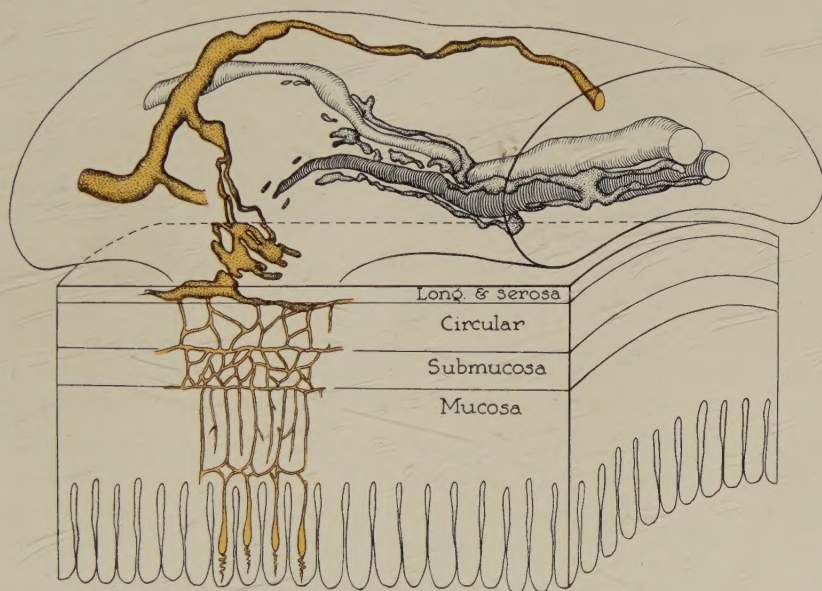


Fig. 3 Diagram showing the relation of portions of the jejunum and omentum involved in an adhesion. Lymphatics are indicated by stippling and blood vessels by vertical lines. Fat (colored yellow) absorbed by the mucosa passes from the intestinal lymphatic plexuses through newly formed connections in the adhesion into an omental lymph channel.

strips. Microscopic study of frozen sections of these strips verified the presence of colored chyle in lymphatic vessels of the lateral type.

4. The course of absorbed fat from the jejunal mucosa to omental lymph vessels is shown in figure 3. Chyle follows its normal pathway from the central lacteal to the lymphatic plexuses of the longitudinal muscle and serosa. From this

area it passes through the newly formed connections of the jejunal-omental adhesion into the omental lymph channels. In the subsequent course it traverses the omental lymph node and reaches the omental pole of the duodenal node.

5. Our experiments prove that omental lymphatics in omental-jejunal adhesions can assume the function of lacteals in transportation of chyle.

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REVIEWS OF APPROVED FILMS IN ANATOMY AND BIOLOGY

The following films in biology have been approved by one or more members of the Committees of the American Association of Anatomists or the American Society of Zoologists, and The Wistar Institute Committee.

Committee of the American Association of Anatomists:

Eliot R. Clark, Warren H. Lewis, Carl C. Speidel, and consultants.

Committee of the American Society of Zoologists:

Ralph Buchsbaum, Robert Chambers, Oscar W. Richards, and consultants.

Committee of The Wistar Institute of Anatomy and Biology:

Edmond J. Farris, Cranford Hutchinson, John S. Nicholas, and others as appointed.

EARLY DIVISION, 2-8 CELLS, OF LIVING MONKEY EGG IN VITRO

WARREN H. LEWIS AND CARL G. HARTMAN

(80 feet, monochrome, silent)

This brief film constitutes a most valuable record, for it shows the divisions, from 2 to 8 cells, of a living *monkey* egg cultivated in vitro. While Doctor Lewis' film showing the early development of the rabbit egg is much more extensive, this film is remarkable because of the far greater difficulty involved in obtaining the material. It represents an important step in the modern renaissance of embryology, in which the *living* embryo is studied. The photography is excellent.

★A — E. R. CLARK.

DIVIDING CANCER CELLS IN VITRO

WARREN H. LEWIS AND M. R. LEWIS

(160 feet, monochrome, silent)

This exquisite film shows a number of mitotic divisions of cells in tissue cultures grown from tissue taken from malignant tumors. It shows many details of the activity of dividing cells which would have escaped notice without the aid of the time-lapse motion picture. There are two triploid divisions. The film forms valuable research material as a record of the behavior during division of malignant cells which may be compared with that of normal cells. The records of source of material and of timing are given completely.

★A — E. R. CLARK.

GROWTH OF CAPILLARIES AND ENDOTHELIUM FROM THE
SUBCUTANEOUS TISSUE OF 7-DAY CHICK EMBRYOS
IN 2-DAY TISSUE CULTURES IN LOCKE-
BOUILLON-DEXTROSE-MEDIUM

WARREN H. LEWIS

(140 feet, monochrome, silent)

In this reel Doctor Lewis has succeeded in obtaining pictures which present the extension, in tissue cultures, of blood-vascular endothelium. It shows the migration outward from the explanted tissue, of endothelial cells which tend to remain connected with one another, and to anastomose, forming networks. Small vacuoles appear in the cells, but typical blood-capillary lumina fail to develop. Thus the film constitutes a valuable contribution to the question of the inherent growth properties of blood-vascular endothelium in the absence of blood-pressure, blood-flow and interchange of substances through the capillary wall. ★A — E. R. CLARK.

SEEING THE UNSEEN — HIGH SPEED MOTION PICTURES
TAKEN WITH STROBOSCOPIC LIGHT — 2 REELS

H. E. EDGERTON, K. J. GERMESHAUSEN AND H. E. GRIER

(800 feet, monochrome, silent)

Producer: Dept. Electrical Engineering, Massachusetts Institute Technology

The first part of reel no. 1 shows the construction of the original apparatus designed by scientists of the Massachusetts Institute of Technology, by which motion pictures may be taken at rates as high as 70 feet per second. The motion of the film is continuous, the light from a mercury vapor lamp being turned on and off by a commutator attached to the camera. When the film is projected, motion may be retarded to one-seventieth natural speed.

The remainder of reel no. 1 and all of reel no. 2 present pictures which have been taken with the apparatus of a wide variety of phenomena, such as the bursting of soap bubbles, birds in flight, the shattering of a light bulb, the flight of a mosquito and a housefly, the deformation of a foot-ball when kicked and of a rubber ball when bounced, the volcanic-eruption-like reaction to milk drops, the breaking of a cup of milk, and the action of arm and leg muscles. In all cases the enormous slowing up of the action brings out features undetectable at normal speeds.

The apparatus is clearly of great value for the analysis of many types of action too rapid for the eye to follow, and the picture is useful in demonstrating the machine and its possibilities. ★D — E. R. CLARK.

★ Copy deposited in the Film Depository of The Wistar Institute, and is available to all scientists for use at The Wistar Institute.

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